universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

„The maintenance of neuronal polarity in aging *Drosophila melanogaster* “

Verfasserin

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angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag.rer.nat.)

Wien, 2011

Studienkennzahl lt. Studienblatt:	A 490
Studienrichtung lt. Studienblatt:	Diplomstudium Molekulare Biologie
Betreuer:	Univ.Prof. Dr. Michael Kiebler

Abstract

A fundamental feature of neuronal cells is that they possess a highly polarized morphology, characterized by a single long axon and multiple short dendrites. This polarized morphology and the proper localization of proteins and other subcellular constituents form the basis for neuronal function and their assembly into functional neuronal networks. [1-3] Specifically, the selective targeting of proteins to specialized subcellular domains, such as the axon, is of central importance for the establishment and maintenance of these molecularly and functionally highly specialized subcellular compartments. While neuronal polarity and respectively the polarized distribution of proteins has been thoroughly studied in the early stages of life in mammalian organisms, it is less well characterized in invertebrates and the late stages of life.

Drosophila melanogaster has served as an excellent model for studying the establishment of cell and neuronal polarity. Only recently, it has become clear that *Drosophila* neurons exhibit a clear molecular polarization, apparent by the polarized distribution of proteins to either axons or dendrites. Taken together with the appealing properties of *Drosophila* as a model organism, the vast number of available genetic tools and the possibility to study cellular processes in vivo, *Drosophila* represents a convenient, simple and genetically tractable model organism to study the intricate aspects of the establishment and maintenance of neuronal compartments.

In this study we used *Drosophila melanogaster* to validate the polarized distribution of proteins to the axonal compartment by the selective expression of transgenic fluorescent proteins with presumed axonal localization in the mushroom bodies of young adult flies. We could prove that several aspects of neuronal polarity, such as the selective targeting and trafficking of membrane and transport proteins to the axonal compartment are established early in the life of *Drosophila* neurons. Although some aspects of neuronal polarity may be less well established the early stages of life, our findings substantiate the use of *Drosophila* as a model to study the complex processes of neuronal polarity.

Secondly, we aimed to investigate the maintenance of the polarized distribution of proteins with age by means of a comparative in vivo screen. Mechanisms that underlie the aging process and contribute to the commonly observed age-associated cognitive decline are ill defined. Indications that alterations in the polarized distribution of

proteins may occur in the aged brain and contribute to the aging process come from the observations of Niewiadomska and colleagues, who could demonstrate the age-dependent redistribution of axonal microtubule binding proteins, affecting axonal transport in mammals [4-6]. Expressing axonal proteins in the mushroom bodies of young and aged flies, we observed alterations in the distribution of two membrane proteins, whereas the localization of a related membrane protein was unaffected, suggesting that the observed rearrangements are not a consequence of the long-term expression of transgenic proteins. Transport proteins were found to maintain their highly selective localization to the axonal compartment, indicating no general impairment of axonal trafficking. Moreover we found indications that presynaptic proteins become polarized late in neuronal development, after neuronal compartments are already morphologically distinguishable. Our findings raise the possibility of re-localization of certain axonal proteins in the aged central nervous system, but further research regarding endogenous proteins and underlying mechanism is required.

Zusammenfassung

Ein grundlegendes Merkmal neuronaler Zellen ist ihre stark polarisierte Morphologie, welche sich in der Ausbildung eines einzigen langen Axons und mehrerer kurzer Dendriten manifestiert. Diese polarisierten Morphologie, sowie die differentielle, polare Lokalisation von Proteinen und anderen subzellulären Bestandteilen bilden die Grundlage für die Funktion von Nervenzellen und deren Vernetzung zu funktionellen neuronalen Netzwerken. [1-3] Insbesondere die selektive Lokalisation von Proteinen innerhalb spezialisierter subzellulärer Bereiche, wie dem Axon, ist essentiell für die Etablierung und Aufrechterhaltung dieser molekular und funktionell hoch spezialisierten subzellulären Kompartimente. Während neuronale Polarität, bzw. die polarisierte Verteilung von Proteinen, in jungen Säugetierneuronen relativ gut charakterisiert wurde, ist wenig über diese Aspekte in Invertebraten und alternden Neuronen bekannt.

Drosophila melanogaster hat sich als hervorragender Modelorganismus zur Erforschung der Etablierung zellulärer und neuronaler Polarität erwiesen. Erst kürzlich konnte gezeigt werden, dass *Drosophila* Neuronen eine klare Polarität auf molekularer Ebene aufweisen, welche sich in der polarisierten Verteilung von Proteinen entweder im Axon oder den Dendriten zeigt. Dies, sowie die praktischen Vorteile von *Drosophila* als Modellorganismus, die Vielzahl an verfügbaren genetischen Werkzeugen und die Möglichkeit zelluläre Vorgänge in vivo zu beobachten, machen *Drosophila* zu einem unkomplizierten und genetisch einfach manipulierbaren Modellorganismus für die Erforschung komplexer Aspekte der Etablierung und Aufrechterhaltung neuronaler Kompartimente.

In der vorliegenden Studie nutzten wir *Drosophila* melanogaster um die polarisierte Verteilung von Proteinen in Axonen zu bestätigen. Durch die selektive Expression transgener fluoreszierender Proteine mit vermeintlich axonaler Lokalisation im Pilzkörper von jungen Fliegen konnten wir nachweisen, dass einige Aspekte neuronaler Polarität, wie die selektive Lokalisierung von Membran- und Transportproteinen im axonalen Kompartiment, bereits in jungen Drosophila Neuronen etabliert sind. Obwohl manche Aspekte der neuronalen Polarität in jungen Neuronen weniger gut etabliert zu sein scheinen, bestätigen unsere Ergebnisse die Eignung von *Drosophila* als Modellsystem, um die komplexen Prozesse neuronaler Polarität zu untersuchen.

Des Weiteren untersuchten wir die Aufrechterhaltung der polarisierten Verteilung von Proteinen im Alter, mittels eines vergleichenden *in vivo* Screens. Hinweise darauf, dass Veränderungen in der polarisierten Verteilung von Proteinen im alternden Gehirn auftreten können und zu dessen Alterungsprozess beitragen, gehen aus den Arbeiten von Niewiadomska und Kollegen hervor, die zeigen konnten das es zu altersabhängigen Veränderungen in der Lokalisation axonaler Mikrotubuli-bindender Proteine, und als Konsequenz Veränderungen im axonalen Transport [4-6]. Durch die transgene Expression axonaler Proteine in den Pilzkörpern junger und alter Fliegen konnten wir Veränderungen in der Verteilung zweier axonaler Membranproteine zeigen, während die Lokalisation eines verwandten Proteins erhalten blieb. Dies deutet darauf hin, dass die beobachteten Veränderungen nicht das Ergebnis der langzeitigen Expression transgener Proteine sind und der Funktionalität des angelegten Screens. Die axonale Lokalisation von Transportproteinen blieb erhalten und weist darauf hin, dass es zu keinen allgemeinen Beeinträchtigung des axonalen Transports kommt. Des Weiteren fanden wir Hinweise darauf, dass präsynaptische Proteine in der neuronalen Entwicklung ihre akkurate, polarisierte Lokalisation erst etablieren, nachdem neuronale Kompartimente morphologisch klar unterscheidbar sind. Unsere Ergebnisse deuten darauf hin, dass es zur Umverteilungen spezifischer axonaler Proteine im gealterten zentralen Nervensystem kommen kann, aber um diese Fragestellung endgültig zu klären sind fortführende Forschungen hinsichtlich endogener Proteinen und der zugrunde liegenden Mechanismen erforderlich.

1.Introduction

1.1. Neuronal polarity and compartmentalization

More than a 140 years ago Otto Dieters firstly coined the basic concept of neuronal polarity based on the highly specific morphology of neurons. He described the emergence of „a variable number of highly branched, thin protoplasmic processes from the cell body...", today's dendrites, and a „prominent single protoplasmic process...", which he termed as „the axis cylinder", nowadays know as the axon. Thirty years later this basic concept was developed further by Cajal into the functional concept of neuronal polarity, referred to as „The Law of Dynamic Polarization". Cajal suggested that the transmission of a nerve impulse occurs from dendrites and the cell body to the axon, with the dendrites and cell body functioning as a „receptor apparatus", whereas the axon represents an „apparatus of emission and distribution". Although some exceptions from the Law of Dynamic Polarization have to be considered, this fundamental concept is still regarded the basis for neuronal and consequently brain function. [2]

Today, it is well established that neurons are highly polarized cells, which form three primary subcellular compartments; axon, dendrites and cell body. Beyond this general partition, subcellular compartments can be divided further into highly specialized regions, such as the axonal initial segment at the base of the axon, which is characterized by the concentration of voltage-gated sodium channels and serves as a point for integration of incoming signals and action potential initiation [7].

The establishment and maintenance of these distinct subcellular compartments is known to be fundamental for neuronal function, with each subcellular compartment taking over a specialized role, as the dendrites receive and integrate incoming signals, whereas the axon is responsible for further processing and transmittance of incoming information. [2]

In addition to these diverse functional features, dendrites and axons have distinct morphological features. Typically, a neuron possesses a single long axon that emanates from the cell body (to transmit information) and several shorter, branched dendrites (to receive information). Axons and dendrites differ in caliber at the base of their emergence. Dendrites are usually thinner than axon and decrease in diameter with branching, while the caliber of axons remains constant. [2]

Apart from these general morphological and functional features, dendrites and axons exhibit clear structural and molecular differences. Several cellular constituents localize to distinct subcellular domains, for instance proteins that are essential for neuronal function are differentially targeted to axons or dendrites. [2]

Some intracellular organelles exhibit a differential subcellular localization, for example late endosomes and the Golgi apparatus are restricted to the cell soma and proximal dendrites. Polyribosomes are dispersed throughout the somatodendritic compartment, but excluded from axons. mRNAs are predominately found in the cell body, with some exceptions that are specifically targeted to dendrites [2,8]. Other organelles, such as the endoplasmatic reticulum and mitochondria are distributed throughout the cell. Mitochondria might represent a special case, since their localization is known to be highly dynamic and differential localization of mitochondria was observed under certain conditions [9]. Differential localization of mitochondria might be required to specifically meet the metabolic demands of certain subcellular sites [2], e.g. the clustering of mitochondria was observed at sites of increased synaptic transmission, which requires a high level of ATP and Ca2+ buffering for efficient synaptic transmission [10].

Synaptic components specifically localize to axons or dendrites, where they cluster at sites of synaptic contact. Predominantly, axons contain specialized presynaptic structures, including synaptic vesicles, vesicle associated proteins and voltage gated ion channels, whereas dendrites contain postsynaptic components, such as neurotransmitter receptors and specialized postsynaptic densities [2,3,11]. The highly polarized distribution of these components is thought to be governed by two distinct mechanisms; first, an endogenous targeting of synaptic constituents to dendrites or the axon and secondly their subsequent clustering at synaptic sites, triggered by cell-cell contact [2,3,11].

Neuronal polarization is also reflected on the level of the cytoskeleton, by a differential organization of microtubules, specific localization of cytoskeleton associated proteins and differential phosphorylation states of cytoskeletal components. In the axons, microtubules display an uniform polarity with their plus-ends distal to the cell body, whereas in proximal dendrites microtubules are of mixed polarity [12,13]. Microtubule binding proteins, involved in the organization and stabilization of the microtubule cytoskeleton polarize to axons or dendrites, such as the microtubule-binding

protein MAP2 that is concentrated in the somatodendritic compartment excluded from the axon, while dephospho-Tau is concentrated in axons [14,15]. These differences in the axonal and dendritic microtubule cytoskeleton are thought to facilitate polarized trafficking to axons and dendrites, as they may serve as landmarks for the sorting of dendritic and axonal constituents [1]. Other cytoskeletal components, such as neurofilaments are present in axons and dendrites, but are enriched and highly phosphorylated in the axon [2].

Another essential molecular feature that neurons share with other polarized cells is the ability to selectively target proteins, regardless of being localized to the plasma membrane or elsewhere, to distinct subcellular compartments, respectively axons or the somatodendritic compartment [3,16]. Especially membrane proteins exhibit a highly polarized pattern of distribution. For example voltage gated Na+, K+ and Ca2+ channels are specifically targeted and retained at certain subcellular microdomains, which might allow neurons to establish specialized sites with functionally unique properties. [2,17]

Taken together, these molecular differences between axons and dendrites are fundamental for neuronal polarity and might provide cues for axonal/dendritic identity.

1.1.1. Mechanisms underlying neuronal polarity

Little is known about cellular mechanisms determining the compartmentalization and polarization of neurons. Undoubtfully, segregation, selective transport and trafficking of intracellular components, such as organelles, RNA and proteins, is of great importance for the establishment and maintenance of neuronal polarity. Moreover, accurate, polarized distribution of intracellular components is crucial for neuronal function, since it serves as a primary mechanism to establish and maintain the identity of highly specialized subcellular domains. [2] For its importance in the given study I will focus in the following on the segregation and selective transport of proteins to distinct compartments, although selective trafficking of RNA and organelles might be equally important. The maintenance of the polarized distribution of proteins is exemplified in the following section, by the polarized trafficking of membrane proteins, which is among the best understood mechanisms for maintaining neuronal polarity.

1.1.2. Polarized trafficking of Membrane proteins

Membrane proteins, including neurotransmitter receptors, ion channels, and adhesion proteins, differentially localize to axons or dendrites and their selective targeting to distinct subcellular sites is among the best understood mechanisms for polarized protein sorting [2,18]. Significant impact on understanding neuronal membrane protein sorting came from studies exploiting methods previously applied to study protein sorting in epithelial cells. Several of these studies could show significant parallels between protein sorting in epithelial cells and neurons [2]. By monitoring the distribution of endogenous proteins or introducing exogenous proteins by viral infection in neurons and epithelial cells, these studies could proof that several proteins exhibit a polarized behavior in both cell types, such as viral proteins VSV-G and SFV-E, neuronal GABA receptors or transferrin receptors [2,19-25]. Although a few proteins that were found to be polarized in epithelial cells did not polarize in neurons and vice versa, it can be reasonably assumed that neurons and epithelial cells share fundamental mechanisms for the selective targeting of proteins [2,16]. Thus, the polarized trafficking of membrane proteins in neurons is assumed to involve the following, congeneric steps: (1) Sorting of axonal and dendritic proteins into divers populations of carrier vesicles, (2) targeted transport to the destined sites, (3) insertion into distinct membrane domains and (4) stabilization and anchoring within the membrane. [2]

Sorting of axonal and dendritic membrane proteins presumably occurs in the trans Golgi network (TGN), where membrane proteins are segregated and incorporated into different populations of transport vesicles [2,26,27]. Segregation and selective incorporation into vesicles is dependent on critical sorting sequences, which widely differ between different proteins and are rather heterogeneously located within the given proteins [3]. Mechanisms that govern membrane protein sorting are in general ill-defined, but several mechanisms were proposed to be involved in this process, including the association with distinct lipid types, receptor-mediated sorting and regulation by G proteins, such as the small G proteins of the Rab family [2,28].

Subsequent transport of the vesicles to their destination is likely to be dependent on the microtubule cytoskeleton, but the actual mechanisms remain nebulous. Differential affinity of distinct types of vesicles to certain motor proteins might provide a partial explanation. Firstly, vesicles destined for the axon may have a higher affinity to plus-end targeted motors, whereas vesicles destined for dendrites may preferentially as-

sociate with minus-end targeted motors. Together with the given differences in the organization of microtubules in axons and dendrites [12,13,28], this difference in motor protein affinity may contribute to an initial sorting of trafficking and synaptic vesicles [1,2,28]. Furthermore, the high diversity of motor proteins and associated adapter proteins may provide an additional level of regulation for the targeting of distinct types of vesicles and other cargos to their destination [1,28].

After arrival of trafficking vesicles near their target site within the membrane, several mechanisms are hypothesized to facilitate the highly regulated fusion of the vesicles with their destined acceptor membrane, but the underlying mechanisms remain ill defined. The high fidelity of this process might be depended on the existence of specialized microdomains within the membrane, which are characterized by their association with specialized docking proteins, which in turn may interact with vesicle associated proteins, e.g. Rab or coat proteins, and trigger vesicle docking and fusion [1,2,18].

Once integrated into the membrane, lateral diffusion of membrane proteins needs to be limited in order to stabilize the proteins at their site of action or/and destined subcellular compartment. In the axon, the latter is assured by the presence of a dense membrane undercoating at the axonal initial segment, which is widely believed to act as a diffusion barrier and helps to maintain distinct axonal and somatodendritic plasma membrane properties [29]. Furthermore diffusion of some membrane proteins might be limited by association with the cortical cytoskeleton via specific spectrin/ankyrin isoforms, as observed in epithelial cells [1,2,28].

Apart from these general mechanisms for targeting and retaining proteins at their destination, other processes such as local protein synthesis, site-specific post-translational modification, and local degradation of proteins may contribute to the differential localization of proteins to axons or dendrites [2].

1.1.3. The role of active axonal transport for the maintenance of neuronal polarity

Most dendritic and axonal proteins are synthesized in the cell body and have to be transported over long distances to their final destination, respectively axons and dendrites. How proteins are sorted for dendritic or axonal transport is widely unclear. Several proteins were observed to be bidirectionally transported along microtubule

tracks in association with membranous organelles at different speeds [1]. These proteins are thought to associate with specific microtubule binding motor proteins in the cell body, which actively transport them along microtubule tracts. Two major classes of microtubule binding proteins, kinesins and dyneins, are thought to mediate long range microtubule-based transport and govern the directionality of transport [1,2]. Most members of the large protein superfamily of kinesins travel to the growing end of microtubules (the so called plus (+) end), whereas multimeric complexes composed of cytoplasmic dynein, dynactin and associated protein move towards the non-growing minus (-) end. This intrinsic property of motor proteins is thought to contribute to the initial sorting of dendritic and axonal proteins. Association of cargos with the dynein complex may determine its delivery to dendrites, since dynein can enter dendrites as a consequence of the mixed polarity of microtubules in the proximal partition of the dendrites [12,13]. In contrast, microtubules in the axon are monopolar with their (+) ends projecting towards the periphery [12,13], hence association of cargo with (+) end traveling kinesins might facilitate its delivery to the axon [1,2]. A variety of different kinesins and dynein complexes, which differ in the composition of accessory subunits, has been identified and is thought to specifically associate with different cargoes, adding an additional level of specificity to motor protein-dependent trafficking [1]. How cargo dissociates from motor proteins and how e.g. cytoplasmic proteins are retained once they arrive at their destination still needs to be clarified.

1.1.4. Maintenance of neuronal polarity and polarized protein distribution with age

Once established, little is known about the maintenance of neuronal polarity. In general there is no evidence for changes in the polarity of neurons under normal physiological conditions. Nevertheless, experimental evidence exists that at least during development neurons are capable of changes in their polarity in response to injury [2,30]. It is not known wether similar switches in polarity occur during normal development or in the adult nervous system.

Interestingly, changes in polarity are thought to be associated with some age-related neurological diseases [2]. Among these is Alzheimer‘s disease, where alterations of the dendritic and axonal microtubule cytoskeleton and associated proteins are con-

sidered hallmarks of the disease [31-33]. Up to date, alterations of the neuronal cytoskeleton and changes in the distribution of cytoskeleton associated proteins during normal aging are only vaguely defined, but Tubulin, Tau and a recently discovered microtubule associated protein (MAP) CacyBP/SIP were found to change their localization with aging. While being predominantly detected in dendritic or axonal processes in young rats, a relocation of these proteins occurs in aged rats and they majorly accumulate in the cell body [4-6].In contrast, another MAP, MAP2, did not significantly change its localization in the same animals, indicating that the redistribution of Tau and CacyBP/SIP were not caused by a general degeneration of cell processes [6] and can be considered an age-associated, molecular rearrangement. Age-dependent relocation of Tau is thought lead to a destabilization of the axonal cytoskeleton, which might cause an impairment of active axonal transport, as exemplified for the retrograde transport of the nerve growth factor (NGF) and its receptors in basal forebrain cholinergic neurons [4,5,34]. Furthermore mislocalization of Tau and its effector GSK-3β and consequent degeneration of the cytoskeleton have been proposed to account for the age-associated degeneration of basal forebrain cholinergic neurons and may represent general mechanisms involved in neurodegeneration and cognitive decline of brain performance during physiological aging [34].

Another indication for age-dependent alterations of polarized trafficking of cellular components comes from the observation that several age associated neurological diseases are associated with abnormalities in cellular trafficking, respectively axonal transport [35]. A slowdown of microtubule associated motor proteins, kinesin-1 and dynein was reported in the early stages of Alzheimer‘s and Parkinson disease. Moreover these alterations were hypothesized to not only contribute, but even trigger neurodegenerative processes, with particular regards to the role of retrograde transport. [35] Wether similar alterations of the intracellular transport occur under physiological conditions in the aging CNS is currently unknown.

1.2. Drosophila melanogaster

1.2.1. *Drosophila* as a model system

The fruit fly, *Drosophila* melanogaster, has firstly been used as a genetic model organism in the beginning of the 20th century by the American embryologist and ge-

netic pioneer Thomas Hunt Morgan. Till today it is among the most popular model organisms in biological and biomedical research. Its basic genetic properties, the convenient experimental handling and the vast number of readily available genetic tools make *Drosophila melanogaster* an optimal model organism for genetics [36]. Although flies and humans vary considerably evolutionary and anatomically, there is a considerable conservation of gene sequences and function, as well as developmental and cellular processes [37]. Genetic studies of the early development of cellular and respectively neuronal polarity [38-42] have made significant contributions to the field, not least because most studies regarding neuronal polarity in mammals have been conducted in primary cell culture, whereas studies in *Drosophila* benefit from the possibility of exploiting the richness of genetic tools and observe neurons in vivo.

1.2.2. *Drosophila* central nervous system

The central nervous system of *Drosophila* has been extensively studied in the past decades and has proven to be a convenient model to study various neurological processes. Morphological and functional properties of many neuronal subpopulations in the fly brain are well characterized, enabling researchers to have recourse to a vast amount of information on each of these subpopulations [43][www.flybrain.org, 11/2010].

Basically the fly brain consists of a central brain, which is laterally connected to the two optic lobes and posterior to the thoracic ganglion. The central brain can be divided into five major brain centers with distinct functions. (1) The antennal lobes, primary neuropils involved in olfactory chemosensory reception. (2) The mushroom bodies, a paired neuropil structure, which primarily receives information from antennal lobe projection neurons and is thought to be involved in higher order olfactory learning and memory. (3) The central body complex, which is composed anteriorly of the ellipsoid body, the fan shaped body and superior arch that lie above the paired noduli. All of the above mentioned neuropils are connected to the protocerebral bridge and the protocerebrum (4), a collection of discrete concatenated neuropils with widely unknown functions. (5) The posterior slope and lateral deutocerebrum, which both include neuropils involved in mechanosensory and visual processing. The lateral deutocerebrum, receives input from the optic lobes, which are required for the

processing of visual input from the compound eye and can be consecutively divided into four neuropils: the lamina, the outer and inner medulla, lobula, and lobula plate [www.flybrain.org, 11/2010].

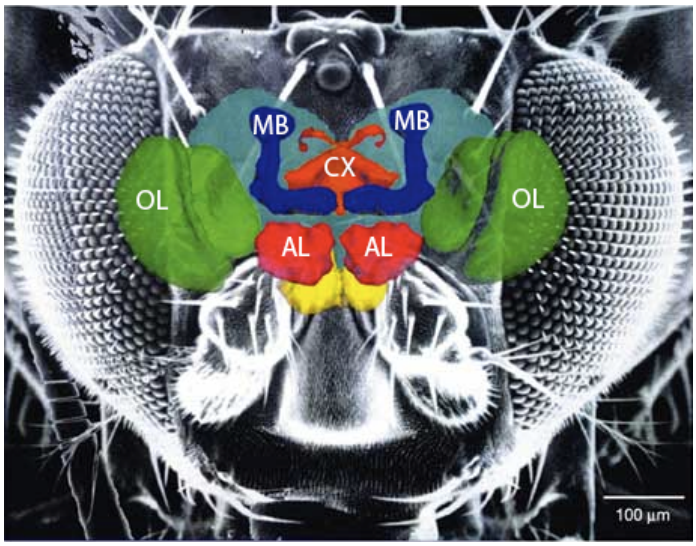


Figure 1: Illustration of the major brain structures in the Drosophila CNS superimposed on a Drosophila head. *Adapted from Martin Heisenberg (2003) Mushroom body memoires: from maps to models. Nature reviews neuroscience 4, pp. 266.*
In green (OL) olfactory lobes; dark blue (MB) mushroom bodies; orange (CX) central complex; red (AL) antennal lobes. Diverse neuropils surrounding the MBs and central complex are indicated in grey. The subesophageal ganglion, which does not belong to the CNS of Drosophila, is depicted in yellow.

Although the fly brain significantly differs morphologically and is of lower complexity, it is composed of the same basic cellular components as the mammalian brain. Neurons and glia cells of the *Drosophila* CNS share many features with their mammalian counterparts, such as a conserved subcellular organization and neurotransmitter systems, including dopamine acetylcholine, glutamate and GABA [44]. Taken together with the benefits of *Drosophila* as a genetic model organisms, these similarities of the basic architecture of its brain have made *Drosophila* a popular alternative model to mammalian systems for neurobiological research. For instance it has been used to study complex processes, such as memory formation [45], olfactory processing [46] and age-related defects in olfactory memory [47]. In the recent years *Drosophila* has been increasingly used to study different aspects of neuronal compartmentalization and polarity, including the differential distribution of proteins and other cellular components [40-42,48-51].

1.2.2.1. The Mushroom bodies

Due to its relevance for the current study I will focus in the following on the architecture of the Mushroom Bodies (MB). The *Drosophila* Mushroom bodies or corpora pedunculata are one of the most prominent brain structures of the protocerebrum and are thought to be involved in various behavioral and physiological functions, such as higher order olfactory associative learning [52,53]. This paired, characteristically shaped neuropil is comprised of approximately 2500 densely packed parallel intrinsic neurons, the so called Kenyon cells, per brain hemisphere [54,55] and receives input from several thousand associated extrinsic neurons. The cell bodies of the Kenyon cells form quadruple clusters at the dorsal posterior cortex on either side of the central complex and each Kenyon cell provides a single neurite that gives rise to several dendritic branches, which extensively arborize within the neuropil and contribute to the hemispherical calyx beneath the cell body layer. The calyx is thought to harbor structures of postsynaptic specialization [41,56] and represents the major input site of the MB, which receives information from olfactory projection neurons (Figure 2A) [57,58]. Directly beneath the calyx, Kenyon cell processes converge firstly into five presumably axonal tracts that subsequently converge to a pair surrounded by a ring of axons and further converge, giving rise to a densely packed tract of axons, the so called peduncle [54,59]. The axonal peduncle runs down anteriorly and ventrally through the central brain and terminates at the heel region, where the axonal tract diverges into five distinct lobes, of which two project vertically (α and α‘) and three horizontally, towards the midline (β, β‘ and γ) (Figure 2B) [54,60-62]. The lobes of the MB are thought to represent the major output site of Kenyon cells, but have been shown additionally to receive input from extrinsic neurons [59,63,64].

Kenyon cells of the *Drosophila* MBs can be classified into three distinct populations, distinguished by their axonal projections into the different lobes; (1) one population with branched, densely packed axons, forming α and β lobe, (2) another population with bifurcated axons, composing the as well densely packed α‘ and β‘ lobe and (3) a population with unbranched axons that sends its axons to the γ lobe, which form a rather diffuse network (Figure 2C) [62]. These distinct populations of Kenyon cells are developed in a sequential manner, starting in the embryo and accomplished during metamorphosis [65]. The three subtypes of Kenyon cells differ in their gene

expression, use of neurotransmitter systems, connection to extrinsic neurons and behavioral functions [59,66,67].

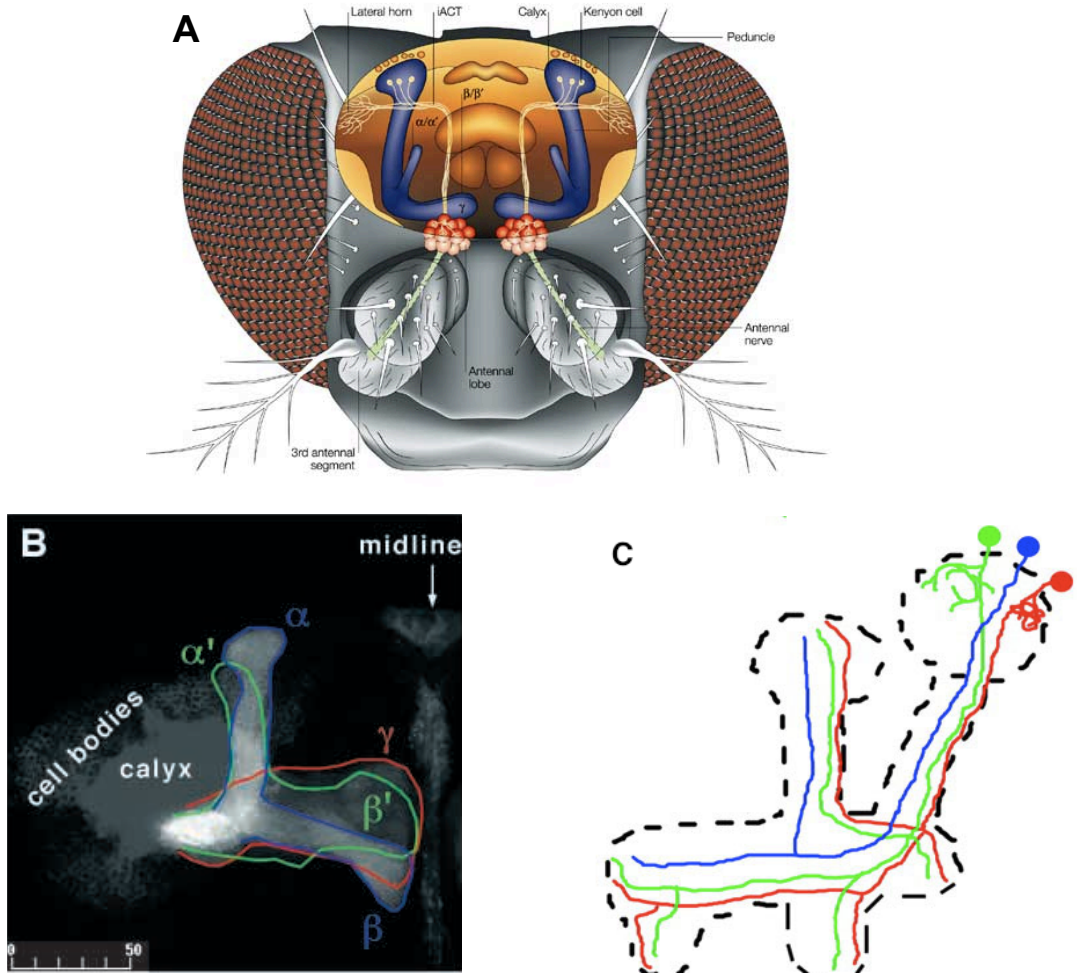


Figure 2: The Drosophila Mushroom bodies

(A) *Illustration of the mushroom bodies with connectivities in the olfactory pathway. From Martin Heisenberg (2003) Mushroom body memories: from maps to models. Nature reviews neuroscience 4, pp.266.* ***(B)*** *Composite confocal images of the close-up view of the right MB of an adult fly, visualized by expression of mCD8- GFP, driven by GAL4-OK107, for the visualization of the whole MB. The five axonal lobes are outlined; γ lobe in red, α′ and β′ lobes in green, and α and β lobes in blue. From Lee et al. (1999) Development of the Drosophila mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast. Development vol. 126 (18) pp. 4065* ***(C)*** *Schematic drawing of the three types of Kenyon cells, classified by their axonal projections. One type of Kenyon cells (green) project their axons only into the γ lobe, another type (blue) with branched axons projecting into α′ and β′ lobes, and the third type (red) as well with branched axons projecting into α and β lobes. From Pauls et al. (2010) Drosophila larvae establish appetitive olfactory memories via mushroom body neurons of embryonic origin. J Neuroscience vol. 30 (32) pp. 10655-66*

1.2.2.2. Polarization of *Drosophila* neurons

Like in other invertebrates, *Drosophila* neurons are typically monopolar and give rise to a single process (Figure 3). Several dendritic branches arise from this single process, which may arborize within the neuropil and contain postsynaptic structures, as to be seen for the *Drosophila* mushroom bodies (Figure 2C, 3B) [2,68]. In some cases, the dendrites of a single cell can contact several different ganglia [2]. It is widely accepted that *Drosophila* neurons possess a clear functional polarization, but it remains highly debated, whether they share structural and molecular characteristics of neuronal polarization with their mammalian counterparts or not [2,40]. Studies that have previously addressed this question led to opposing conclusions. When considering primarily morphological and ultrastructural properties of invertebrate neurons, researchers concluded considerable differences [2,69], whereas studies focusing on molecular properties of different subcellular compartments came to the conclusion that the „major kinds of compartmentalization“ [41] are conserved from *Drosophila* to mammals [41,49,70,71]. Of considerable interest is the recent study of Rolls and colleagues, who could show that the observed functional compartmentalization of *Drosophila* neurons is also reflected on a molecular level. Expressing fluorescently tagged markers and tagged endogenous proteins in larval interneurons of the mushroom body and projection neurons, they could confirm that various manifestations of neuronal polarization and compartmentalization observed in mammals are also true for *Drosophila* [41]. A fundamental manifestation of neuronal polarity, the differential organization of the microtubule cytoskeleton, was affirmed for *Drosophila* neurons, including the differential localization of microtubule binding proteins and polarity of microtubules in axons and dendrites. Tracking of GFP-tagged microtubule plus end binding protein EB1 (EB1-GFP) revealed a similar organization of microtubules as in mammals, with axonal microtubules arranged with their plus ends distal to the cell body, whereas microtubules in dendrites were found to be majorly arranged with their minus ends distal to the cell body, but occasionally dendritic microtubules of mixed polarity were observed [41], although other reports had previously shown that RNA granules are present in dendrites [48]. This contrasts the observed extension of the proteins synthesis machinery into proximal dendrites in mammals. As in mammalian neurons, pre- and postsynaptic connections were detected in specific regions of MB neurons and postsynaptic markers were found to be restricted to the dendritic com-

partment. Synaptic vesicle markers were found to localize to dendrites and axons of the MB, indicating the possibility of dendro-dendritic connections [41]. Moreover, the authors proposed that *Drosophila* neurons might share another feature with their mammalian counterparts and appear to possess a domain of subcompartmental specialization in the axon, the axon initial segment [41]. Additionally the selective localization of non-synaptic membrane proteins, as observed in mammals, was confirmed for neurons of the *Drosophila* CNS [41]. The highly polarized distribution of membrane proteins was additionally confirmed by Katsuki and co-workers., who could show that Roundabout (ROBO) receptors are specifically targeted and retained in certain domains of the axon [71]. Taken together, these findings indicate that *Drosophila* neurons possess a clear polarity, similar to mammalian neurons and represent a powerful system to study the establishment and maintenance of neuronal polarity.

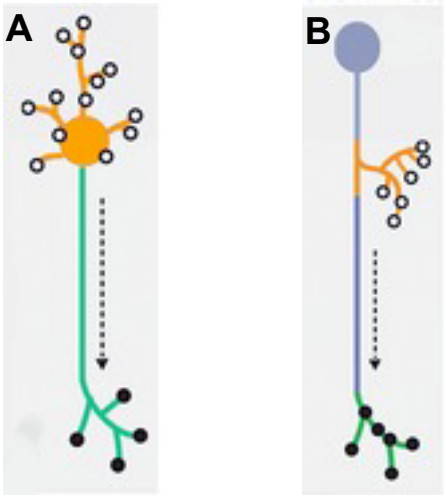


Figure 3: Comparison of a typical vertebrate neuron (A) and a generalized Drosophila neuron (B). *From Spindler and Hartenstein. (2010) The Drosophila neural lineages: a model system to study brain development and circuitry. Development Genes and Evolution vol. 220 (1-2) pp. 1-10.*

Dendritic arbors are depicted in orange and axonal projections in green. **(A)** Multiple, short dendrites emanate from the cell body of a typical bipolar vertebrate neuron and classically harbor postsynaptic sites (white circles), whereas the single *long axon harbors post synaptic sites (black circles).* ***(B)*** *Unlike bipolar vertebrate neurons, Drosophila neurons are unipolar. A single long neurite that emanates from the cell body emits lateral branches, which give rise to dendritic arbors, containing presynaptic specializations.*

2. Experimental readout

2.1.Mushroom bodies as a model neurons

The *Drosophila* mushroom bodies are among the best studied neuropils in the fly brain. Their conserved, layered cellular organization, with a clear subcellular compartmentalization within the layers [41,67,72-75], and the plethora of readily available genetic tools for their manipulation, such as a variety of Gal4 drivers with different strengths that facilitate the expression of target genes throughout the MB or only in distinct lobes (as to be seen in Figure 4A), makes them a forceful tool for genetic and cytological studies. Another major advantage of these model neurons is their known subcellular compartmentalization [41], which has not been shown convincingly for any other neuropil of the fly brain. The large number of Kenyon cells, who's axons run together in the peduncle and are highly concentrated in the lobes, enables the detection of robust axonal phenotypes [76]. Moreover their dendrites are clearly separated from the axons, forming a dense network above the axonal tract (the calyx), which makes the MB an ideal system to study gross alterations in the polarized distribution of proteins [41].

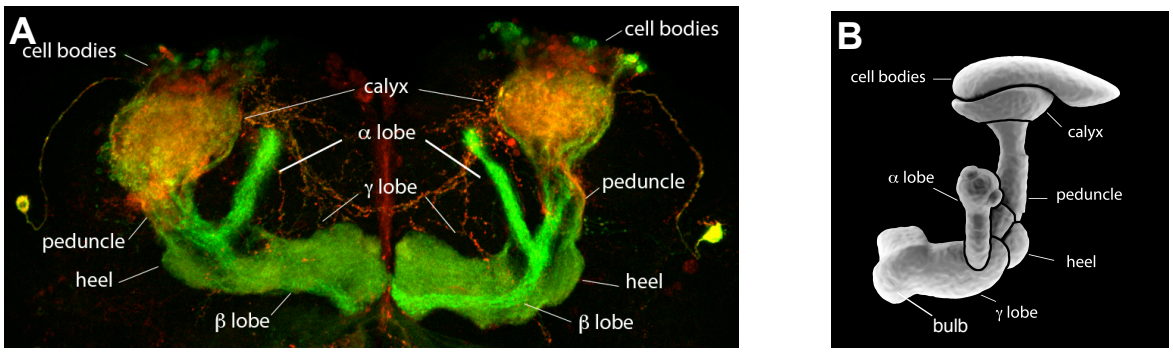


Figure 4: Architecture of the Drosophila mushroom bodies

(A) *Composite confocal images of α, β and γ lobe neurons of the Drosophila Mushroom bodies in adult flies, visualized by 201Y driven expression of the ubiquitous membrane marker, mCD8-GFP (green) and the dendritic marker (DenMark, red). The Genotype of the brain is 201Y Gal4;UAS-DenMark/UAS-CD-8 GFP.*

(B) *Schematic representation of the layered architecture of the MBs with the cell body layer on top, followed by the dendritic calyx. Bellow, proximal axonal tracts converge in the peduncle and split at the heel region into the lobes. Note that for simplicity only the horizontal α and the vertical γ lobe are depicted, as α', β and β' lobe are mostly superimposed by the α or γ lobe.*

2.2. UAS-Gal4 system

The binary UAS-Gal4 system is a frequently used tool for targeted gene expression in *Drosophila* [77]. It is composed of two basic units, firstly the yeast transcription factor Gal4, which can be expressed under the control of a tissue or cell specific promoter and enables the spatiotemporal control of gene expression. Secondly, a target gene of interest fused to a Gal4 responsive Upstream Activating Sequence (UAS). Binding of the Gal4 transcription factor to the UAS activates transcription of the downstream target gene, which is inactive in the absence of Gal4. By crossing flies carrying the Gal4 transcription factor with flies carrying a UAS-target gene of interest, progeny is obtained that expresses the gene of interest. If a tissue-specific promoter controls expression of Gal4, it will result in an identical expression pattern of the UAS-target gene in the progeny. The principle of the Gal4 system is illustrated in Figure 5, as applied in the current study.

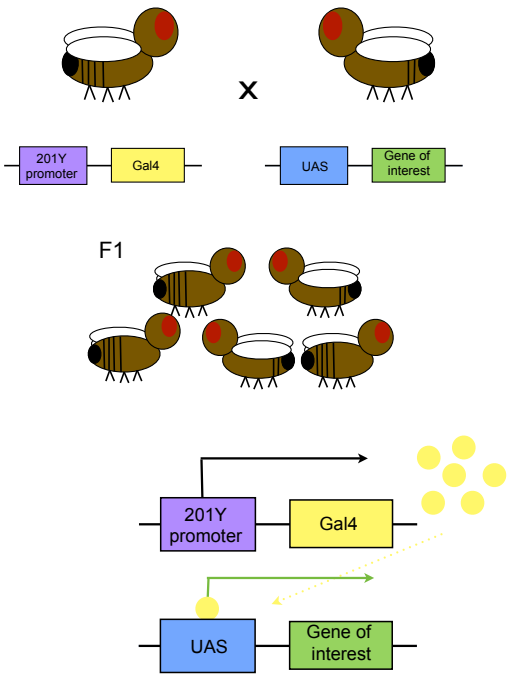


Figure 5: Illustration of the Gal4 system *(adapted with slight variations from Brand and Perrimon. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development (1993) vol. 118 (2) pp. 401-415)*

Transgenic flies that express the yeast transcription factor Gal4 under the control of the 201Y promoter in a subset of MB neurons are crossed to transgenic flies, in which the expression of a GFP tagged protein of interest is controlled by the Gal4 interacting UAS element. The obtained, double transgenic F1-progeny will specifically express the gene of interest in a subset of mushroom body neurons.

A variety of well characterized fly strains carrying the Gal4 transcription factor under the control of different promoters has been generated by research groups worldwide and are publicly available from stock centers. Same accounts for various target genes under UAS control. This powerful genetic tool can be easily combined with other techniques to study gene functions and properties in vivo. It is possible to express fluorescently tagged proteins by the use of the UAS-Gal4 system in order to determine the subcellular localization of a protein of interest. When used in conjunction with high resolution confocal microscopy it is even possible to monitor expression and localization in whole-mount preparations. A recent study has used this approach to characterize the dendritic compartment of four independent neuronal populations, by the means of expressing a novel dendritic marker, named DenMark (Dendritic Marker) with the UAS-Gal4 system. DenMark, a hybrid protein composed of the mouse protein ICAM5/Telencephalin and the red fluorescent protein mCherry, was shown to reliably localize to the dendritic compartment of probably all *Drosophila* neurons [78], suggesting that Gal4 expressed ICAM5 protein maintains its subcellular localization. This finding substantiates the use of the UAS-GAl4 system in conjunction with fluorescent labelled proteins for monitoring the subcellular localization of proteins in *Drosophila* neurons and augments the applications of the Gal4-UAS system for cytological studies.

3. Aims

The differential localization of proteins to distinct subcellular compartments is an indispensable feature of polarized cells. Neurons are highly polarized cells and the ability to selectively target proteins to specialized subcellular domains is essential for the establishment and maintenance of neuronal compartmentalization and in consequence neuronal function; to receive and transmit information. The later is assured by the axon and the polarized distribution of proteins to the axon is essential for the integrity of this molecularly and functionally unique compartment. *Drosophila* melanogaster has served as an invaluable model to study various aspects of cell and respectively neuronal polarity [40-42,49,50,70]. Its practical properties, such as a short lifecycle, the disposability of a variety of genetic tools and the possibility to study physiological processes in vivo [36] make *Drosophila* an ideal system to screen for alterations in the polarized distribution of proteins. Although mounting evidence exists that *Drosophila* neurons are polarized at the molecular level [40-42,50], the polarized distribution of cellular components, including organelles and proteins remains to be highly debated. Hence the first objective of this work is to show that proteins specifically localize to the axon in the young *Drosophila* CNS by the means of an in vivo screen for the localization of transgenically expressed fluorescent proteins in the mushroom bodies.

Secondly, as changes in the polarized distribution of proteins and or lipids will have functional consequences, we aimed to study whether age-associated polarity defects occur during the physiological aging process. A decline in brain performances, e.g. learning and memory is commonly associated with aging. Neuron death, which accounts for the functional decline in neurodegenerative disorders, is restricted in normal aging and unlikely to account for the age-dependent decay of brain function [79]. Neurological equivalents of the functional decline in normal aging are ill defined and the mechanisms that trigger or govern this decline remain elusive. Recently, it has been shown that in mammals, axonal and dendritic microtubule binding proteins are redistributed with age and trigger alterations in axonal transport [4-6]. But the question whether cell polarity in general or the polarized distribution of cellular components changes or shows signs of impairment in the aging central nervous system (CNS) and may contribute to the age-associated decline of brain performance has

not been addressed previously. Therefore the second objective of the present work is to analyze whether or not axonal proteins remain polarily distributed in neurons of the aged brain, by the use of a comparative screen for the localization of transgenic proteins in young and aged individuals.

4. Materials and Methods

4.1. *Drosophila* lines and fly husbandry

Transgenic lines carrying 201YGal4, UASp-Aplip1.EGFP, UAS-Cac1-EGFP, UAS-Khc.EGFP, UAS-mCD8::GFP, UAS-mTau-GFP, UASp-YFP.Rab3, UAS-Syt.eGFP, and UAS-unc-104.GFP were obtained from the Bloomington *Drosophila* stock center. UAS–DenMark lines were kindly provided by Bassem Hassan and UAS-GFP.gpi lines were obtained from Suzanne Eaton. UAS-ROBO1-eGFP/TM6b, UAS-ROBO2-eGFP/Cyo and UAS-ROBO3-eGFP/Cyo lines were a gift from Yasushi Hiromi and UAS-brp.GFP,UAS-lacZ/TM6 line was kindly provided by Patrik Verstreken. pUAS-eGFP-HA-synj was donated by Thomas L. Schwarz. All flies were raised on standard fly food at room temperature and aging flies were transferred every three days into a fresh vial.

4.2. Genetic manipulation

For targeted gene expression we used the binary Gal4-UAS system [77]. In our experiments we used 201Y-Gal4, which expresses Gal4 specifically in γ and the core of α/β lobe mushroom body neurons [54,80]. Flies of the genotype: 201YGal4/Cyo; UAS-DenMark/TM3 were created to allow simple breeding of flies that express DenMark and the candidate protein of interest in the mushroom bodies, by the means of crossing these flies to lines that carry an UAS insertion of a protein of interest fused to a green fluorescent protein.

4.3. Immunohistochemistry

Fly brains were dissected in Phosphate Buffer Saline (PBS) and fixed by incubation in 3,7 % Formaldehyde in PBS containing 0,1 % Triton (PBT) for a maximum of 15 minutes. After fixation, brains were washed three times, once directly and two times for 10 minutes in PBT. Subsequently the brains were incubated for 60 minutes at room temperature in protein blocking solution Pax-DG (5% Normal Goat Serum, 1%Bovine Serum Albumin, 0,1% Deoxycolate, 1 % Triton X-100 in PBS), followed by overnight incubation at 4°C in primary antibody solution containing mouse anti-GFP monoclonal 3E6 from Invitrogen(1:500) and rabbit anti-dsRed polyclonal from Clon-

tech (1:500) in Pax-DG. For detection of endogenous Bruchpilot the antibody solution was prepared, using mouse anti-Bruchpilot (nc82, 1:100) (generated by A. Hofbauer [81]) and rabbit anti-dsRed polyclonal from Clontech (1:500). After incubation with the primary antibodies, the brains were washed once directly and four times at room temperature for 15 minutes. Primary antibodies were detected by incubation with appropriate fluorescently tagged secondary antibodies (Invitrogen) diluted 1:500 in PaxDG for two hours at room temperature. Before mounting, the brains were washed once directly and four times for 10 minutes.

4.4. Microscopy and image processing

Confocal microscope image acquisition was performed either with a Olympus FluoView FV1000 confocal laser-scanning system or a Nikon A1R confocal laser-scanning system. If not indicated otherwise, images were acquired with a 40 x oil objective using optimal setting for a maximal signal to noise ratio. Confocal stacks of immunostained brains were recorded at 1μm distance and projection images were generated with ImageJ 1.44a.

5. Results

In order to determine whether proteins are polarily distributed to the axon and maintain their specific localization with aging, we monitored the distribution of exogenous proteins (candidate proteins) in the mushroom bodies (MB) of young and aged flies. Candidate proteins fused to green fluorescent proteins were specifically expressed in subsets of MB neurons, the so called Kenyon cells, by the use the Gal4-UAS system [54,77] and the GFP signal was amplified by staining with GFP antibodies. Subcellular localization of candidate proteins was examined in whole-mount brains by high resolution confocal microscopy and determined for two age groups, representing young and aged flies. Young flies were three to seven days posteclosion. Aged flies had an age of 35 to 45 days, when most of the initial population is still alive, but can be considered reasonably old. Although the compartmentalization of MB neurons has been determined [41], we used a newly developed, genetically encoded dendritic marker, DenMark, to distinguish axons and dendrites. DenMark is a transgenic fusion protein of the mammalian protein Telencephalin/ICAM5 and the red fluorescent protein mCherry, which can be easily expressed by the use of the Gal4-UAS system [78]. DenMark preferentially localizes to the dendritic domain of *Drosophila* neurons [78] and its co-expression with fluorescent labelled candidate proteins simplified the evaluation of the subcellular localization of each candidate.

5.1. Expression of transgenic proteins remains stable in γ lobe neurons of aged flies

Previous analysis using P[GAL4] enhancer-trap approach for GAL4 directed expression of either β-galactosidase (β-gal) or UAS-mCD8::GFP showed that 201Y driven expression majorly occurs in γ and core of α/β lobe neurons of larvae and young flies [54,80]. We considered this selective expression in a subset of MB neurons beneficial for our approach, as α/α‘, β/β‘ and γ lobe frequently overlap in confocal sections, impeding the evaluation of protein localization. Detailed analysis of 201Y driven β-gal expression revealed that in some occasional older individuals (6 weeks posteclosion) 201Y driven expression of β-gal leads to a “normal” staining of the γ lobe neurons, but a complete absence of the staining in α and β lobe neurons [54]. To test whether expression of transgenic proteins with the Gal4-UAS systems

persists during aging with the selected 201Y Gal4 driver and to validate previous findings, we expressed UAS-mCD8-GFP, an ubiquitous membrane marker that strongly labels neuronal processes, in the MB of young and aged flies.

Young flies. 201Y driven expression of mCD8-GFP was majorly observed in the core of α, β lobe and γ lobe neurons, while being absent from α‘ and β` lobe neurons. In consistency with previous reports, mCD8-GFP homogeneously labeled cell bodies, dendrites and axons of these clonally derived subsets of MB neurons [61], revealing the particularities of MB architecture, as can be seen in Figure 6A.

Aged flies. Expression of mCD8-GFP in γ lobe neurons of aged flies remained unchanged, exhibiting an intense fluorescent signal in all subcellular compartments. Expression in α and β lobe neurons was notably reduced or completely diminished in some of the aged individuals, indicating a variation in 201Y expression pattern during the lifetime of the adult. Regardless of the variation in α and β lobe neurons, 201Y driven expression was persistent in γ lobe neurons of aged flies and appeared to be reasonably stable. No aberrations, such as protein aggregates or morphological alterations, as a consequence of persistent or excessive overexpression could be observed. Hence we decided to continue the present study with this driver, focusing subsequent observations of protein localization on γ lobe neurons, as indicated in the scheme to the right in Figure 6A and 6B.

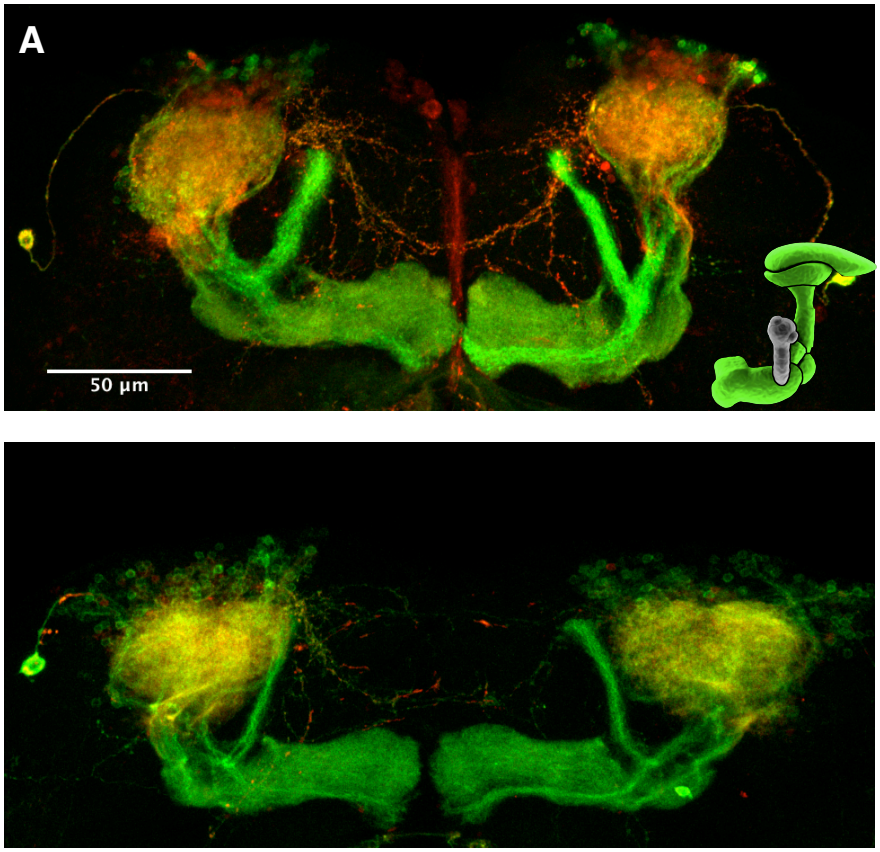


Figure 6: Expression of mCD8-GFP in the MBs young (A) and aged flies (B)

(A) *mCD8-GFP extensively labeled α, β lobe and γ lobe neurons of the MBs in young individuals.* ***(B)*** *mCD8-GFP was notably diminished and occasionally completely absent from α and β lobe neurons of aged flies. Thus, we based subsequent observations of the localization of candidate proteins primarily on γ lobe neurons. The Genotype of the brains is 201Y Gal4;UAS-DenMark/UAS-CD-8 GFP*

Schemes in the lower right corner: Preferential localization of mCD8-GFP in the mushroom bodies is indicated in bright green. As we based our observations of protein localization on γ lobe neurons, localization in α lobe neurons is not indicated.

5.2. Membrane proteins

A hallmark of neuronal polarity is the differential localization of membrane proteins to axons or dendrites. Hence, four fluorescently labelled exogenous membrane proteins, of previously shown axonal polarization, were chosen to determine whether the polarized distribution of membrane proteins is maintained upon aging.

5.2.1. Robo proteins

Roundabout (Robo) proteins are integral transmembrane receptors, belonging to the immunoglobulin superfamily. All three members of the Robo protein family in flies,

namely Robo1, 2 and 3, are highly conserved axon guidance receptors, involved in proper midline crossing of commissural neurons in the spinal cord [82]. During development surface expression of the Robo1, 2 and 3, is highly spatiotemporarly regulated and recent studies in rats showed a persistent basal expression of Robo and its ligands postnatally and even in adulthood [83,84]. Persistent expression of Robo proteins in flies has not been studied yet. However, in *Drosophila*, native and transgenic UAS constructs of eGFP-tagged Robo proteins (Robo-eGFP) were shown to selectively localize to distinct parts of the axon [71]. For their highly reliable axonal localization, the three exogenous hybrid proteins of the Robo receptors and eGFP, namely Robo1-eGFP, Robo2-eGFP, and Robo3-eGFP were expressed in the MB for monitoring the polarized distribution of transmembrane proteins in dependence of age.

5.2.1.1. Relocation of Robo1-eGFP localization to dendrites with age

Young flies. Robo1-eGFP signal was majorly detected in the axons of MB neurons, whereas only a minor signal was abundant in dendrites and the cell body. Contrasting previous reports of Robo1-eGFP localization in vitro, no preferential localization to the proximal part of the axon could be observed, but as the authors stated for larval commissual neurons, localization of transgenic Robo proteins might be less restricted in vivo [71]. However, our data indicates a preferential, uniform localization of Robo1-eGFP to the axon in the MBs of young flies (Figure 7A).

Aged flies. Robo1-eGFP signal was preferentially detected in dendrites of aged individuals, whereas it was notably reduced in axons. Contrasting the findings in young flies, this observation indicates a shift in the axonal to dendritic ratio, with an increase in the dendritic fraction of the protein. This relocation of the signal pattern points to a change in the distribution of Robo1-eGFP, from a preferentially axonal localization in the young to a primarily dendritic localization in the old (Figure 7B).

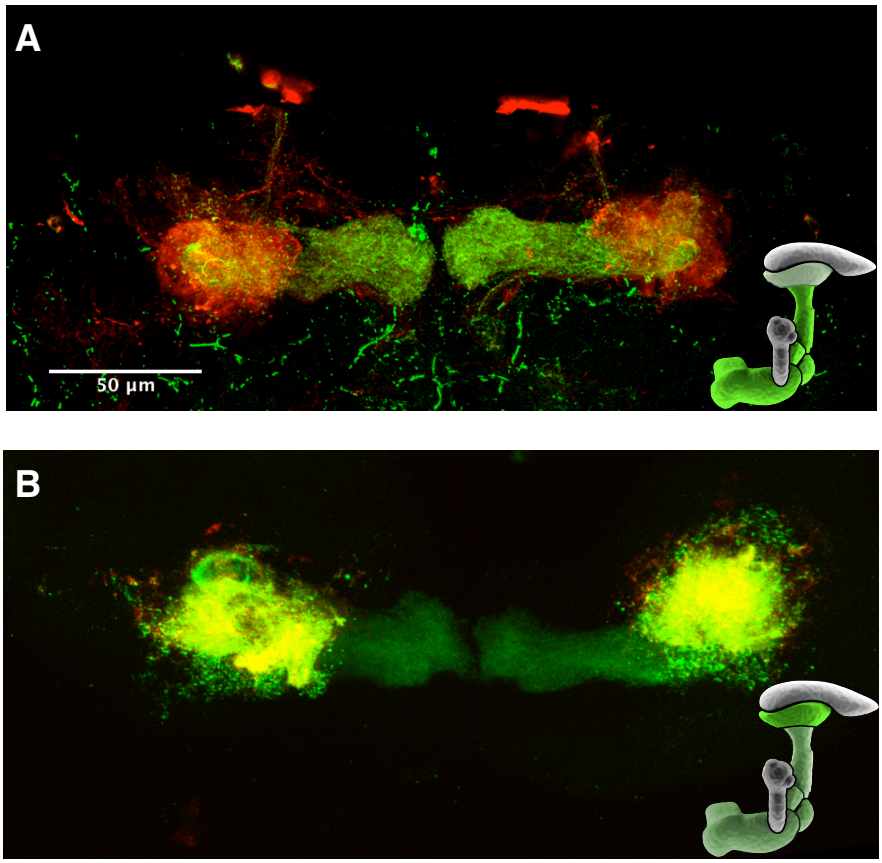


Figure 7: Localization of Robo1-eGFP in the MBs young (A) and aged flies (B)

(**A**) *Robo1-eGFP preferentially localized to the axonal compartment in the MBs of young flies and was weakly detected in dendrites.* ***(B)*** *Robo1-eGFP preferentially localized to dendrites in aged flies and was notably reduced in axons, suggesting a redistribution of the protein with age. Note the complete absence of Robo1-eGFP from α and β lobe neurons in aged flies. The Genotype of the brains is 201Y Gal4;UAS-DenMark/UAS-Robo1-eGFP. Schemes in the lower right corner: Preferential localization of Robo1-eGFP is indicated in bright green and minor localization in faint green.*

5.2.1.2. Preferentially axonal localization of Robo2-eGFP is maintained with age

Young flies. Robo2-eGFP was detected selectively in the axons of MB neurons, while it was completely absent from the cell bodies and dendrites. Robo2-eGFP signal appeared equally strong in distal (lobe) and proximal (peduncle) parts of the axons. But considering the highly concentrated array of axons in the peduncle, which diverge at the heel region and form the more lose network of axons in the γ lobe, it can be reasonably assumed that Robo2-eGFP is rather concentrated in the distal part of the axons, as an equally strong signal was detected in the lobes as in the pe-

duncle, although it contains fewer and more dispersed axons. Thus, Robo2-eGFP was polarily distributed to the axon with a preference for the distal part of the axon, corresponding to previous findings of Katsuki et al. in cultured *Drosophila* neurons [71].

Aged flies. As in young individuals, Robo2-eGFP signal remained selectively localized to the axon, with a minor leakage of the signal in the central part of the dendrites. The preferential localization to the distal part of the axon was less evident than in young flies, which can be contributed to the general increase of the signal as a consequence of persistent expression over the life time of the fly. The major pattern of Robo2-eGFP localization was maintained in aged flies, indicating no changes in the preferentially axonal localization of Robo2-eGFP with age.

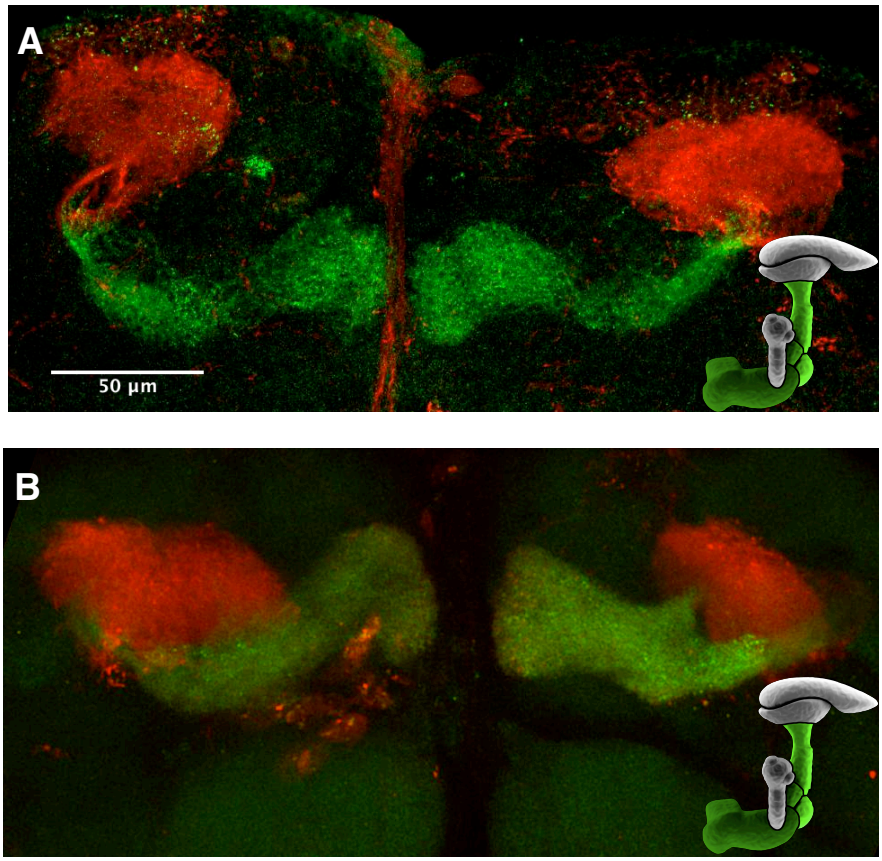


Figure 8: Localization of Robo2-eGFP in the MBs young (A) and aged flies (B)

(**A**) *Robo2-eGFP exclusively localized to the axonal compartment with a concentration in distal parts of the axons in the MBs of young flies.* ***(B)*** *In aged flies, Robo2-eGFP remained preferentially localized to the axons and minor concentrated in distal parts. The Genotype of the brains is 201Y Gal4;UAS-DenMark/UAS-Robo2-eGFP.*
Schemes in the lower right corner: Preferential localization of Robo2-eGFP is indicated in bright green and specific concentrations are shown in dark green.

5.2.1.3. Rearrangement of Robo3-eGFP localization with age

Young flies. In young individuals, Robo3-eGFP was primarily detected in the axons of MB neurons, while being completely absent from dendrites and cell bodies. In some occasional brains, a minor Robo3-eGFP signal was evident in dendrites. Overall the signal pattern resembled Robo2-eGFP signal, but appeared less restricted to the distal part of the axon. Nevertheless, Robo3-eGFP fluorescence was slightly increased in the most distal part of the axons, suggesting in accordance with previous reports, a preferential localization to distal parts of the axon in young flies (Figure 9A) [71]. This preferential localization of Robo2-eGFP and Robo3-eGFP to distal parts of the axon in young flies contrasts the uniformly axonal localization of Robo1-eGFP and might indicate that Robo2-eGFP and Robo3-eGFP indeed selectively localize to certain parts of the axon in the early stages of adulthood.

Aged flies. Compared to young flies, Robo3-eGFP signal was strongly detected in the most proximal part of the axons, equaling the onset of the peduncle, and dendrites. Fluorescence in the shaft of the axons was notably diminished or completely absent, whereas a weak signal was abandoned in the most distal part of the axons, the tips of the γ lobe. Excessive leakage into the dendrites was observed, apparent as discrete, intense spots within the dendritic cloud. Only a weak diffuse signal was present, as to be seen for the dendritic marker, indicating an aggregation of the protein in dendrites. In summary, Robo3-eGFP localization was found to be dramatically rearranged in aged individuals, changing its rather uniform axonal localization with a slight increase in distal parts in the young to an preferential localization to proximal parts of the axons and dendrites in the old (Figure 9B). These observations indicate age-dependent alterations in the localization of Robo3-eGFP.

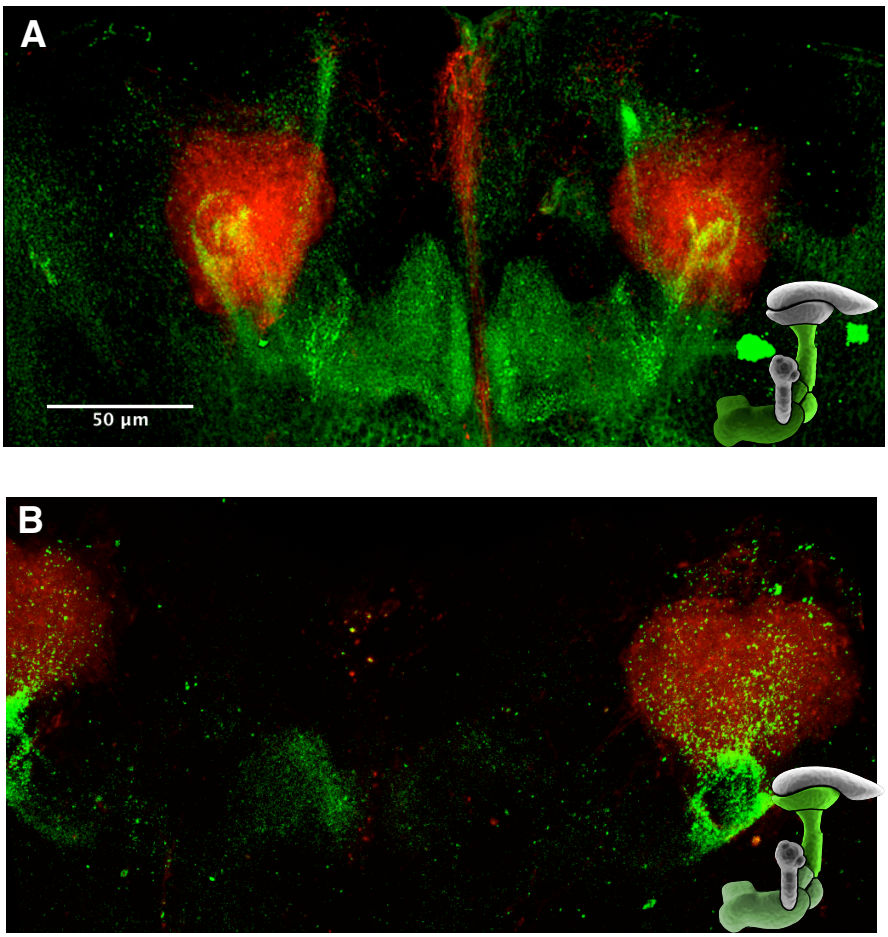


Figure 9: Localization of Robo3-eGFP in the MBs young (A) and aged flies (B)

(A) *In the MBs of young flies,. Robo3-eGFP preferentially localized to the axons with an minor concentration in distal parts.* ***(B)*** *Robo3-eGFP was notably redistributed in the MB of aged flies, as it preferentially localized to dendrites and proximal parts of the axons. The Genotype of the brains is 201Y Gal4;UAS-DenMark/UAS-Robo3-eGFP.*

Schemes in the lower right corner: Preferential localization of Robo3-eGFP is indicated in bright green, specific concentrations in dark green and minor localization in bright green.

5.2.2. Uniform distribution of GFPgpi in the mushroom bodies of young and aged flies

A frequently found posttranslational modification for tethering proteins to the outer leaflet of the plasma membrane is the addition of a glycosyl phosphatidylinositol (GPI) anchor. Dotti and colleagues showed the targeting of GPI-anchored proteins to the axonal domain of primary hippocampal neurons in culture [85] and several endogenous GPI-linked proteins display a conserved axonal polarization in flies and mammals, including certain isoforms of Acetylcholinesterase and the cell adhesion

molecule Fascilin I [86].Direct evidence for axonal targeting of GPI-anchored proteins in *Drosophila* is still missing, but it has been demonstrated that a transgenic GPI-linked UAS-GFP reporter construct, as well as GPI- linked endogenous proteins preferentially localize to specialized micro domains within the plasma membrane, so called lipid rafts [87]. Lipid rafts are sterol and sphingolipid rich domains that are known to be crucially involved in axonal trafficking of proteins [88], potentially indicating axonal targeting of GPI-anchored proteins in *Drosophila* neurons. To test whether GPI-anchored proteins are axonally polarized in *Drosophila* neurons and whether this potential localization is maintained with age, a chimeric protein consisting of GFP with a C-terminally attached GPI-anchor (GFPgpi) was chosen as a representative candidate for studying the localization of GPI-anchored proteins in young and aged flies.

Young flies. GFPgpi signal was uniformly distributed in the membrane of the MB neurons of the young age group and extensively labelled cell body and neuronal processes. No preferential localization to any subcellular compartment could be observed, as to be seen in (Figure 10A). In addition to the extensive staining of the MBs, GFPgpi was detected in an extrinsic brain structure, the outer ring of the ellipsoid bodies, where the signal appeared to be even stronger than in the MBs. Commonly, detection of the Gal4-expressed proteins in extrinsic brain structures points to a basal, Gal4 independent expression of the given UAS-insertion. Hence, we examined whether GFPgpi is expressed at a basal level in the absence of Gal4 in the brain of young flies that carried solely the genomic UAS insertion of GFPgpi. Surprisingly, no expression of GFPgpi was detected in the ellipsoid bodies, but in the MB themselves, indicating that the UAS-GFPgpi insertion might be located in the proximity of a mushroom body specific promoter, which leads to the expression of GFPgpi in the absence of Gal4. Even if not detected in the ellipsoid bodies, the possibility remains that GFPgpi is expressed in the ellipsoid bodies below the necessary levels of detection, which seems rather unlikely judging from the high levels, detected in the presence of the mushroom body specific Gal4 driver. Expressed at basal levels, in the absence of the Gal4 driver, GFPgpi signal uniformly localized to cell bodies, dendrites and axons, suggesting a uniform distribution of GFPgpi in the MB of young flies, regardless of expression levels.

Aged flies. As in the young age group, GFPgpi remained rather uniformly distributed in the MB (Figure 10B), with a general increase of the fluorescent signal in all subcellular compartments. GFPgpi signal appeared to become slightly enriched in proximal axons of the peduncle, but as proximal axons are highly concentrated in this area, this is unlikely to represent a significant concentration of GFPgpi in proximal axons and might be result of the general increase of the fluorescent signal. No fluorescent signal was abandoned in the ellipsoid bodies of aged flies, contrasting the observation in the young. Except for its absence in the ellipsoid bodies, GFPgpi appeared to remain homogeneously distributed in the membrane of MB neurons, suggesting no alterations in the distribution of this protein.

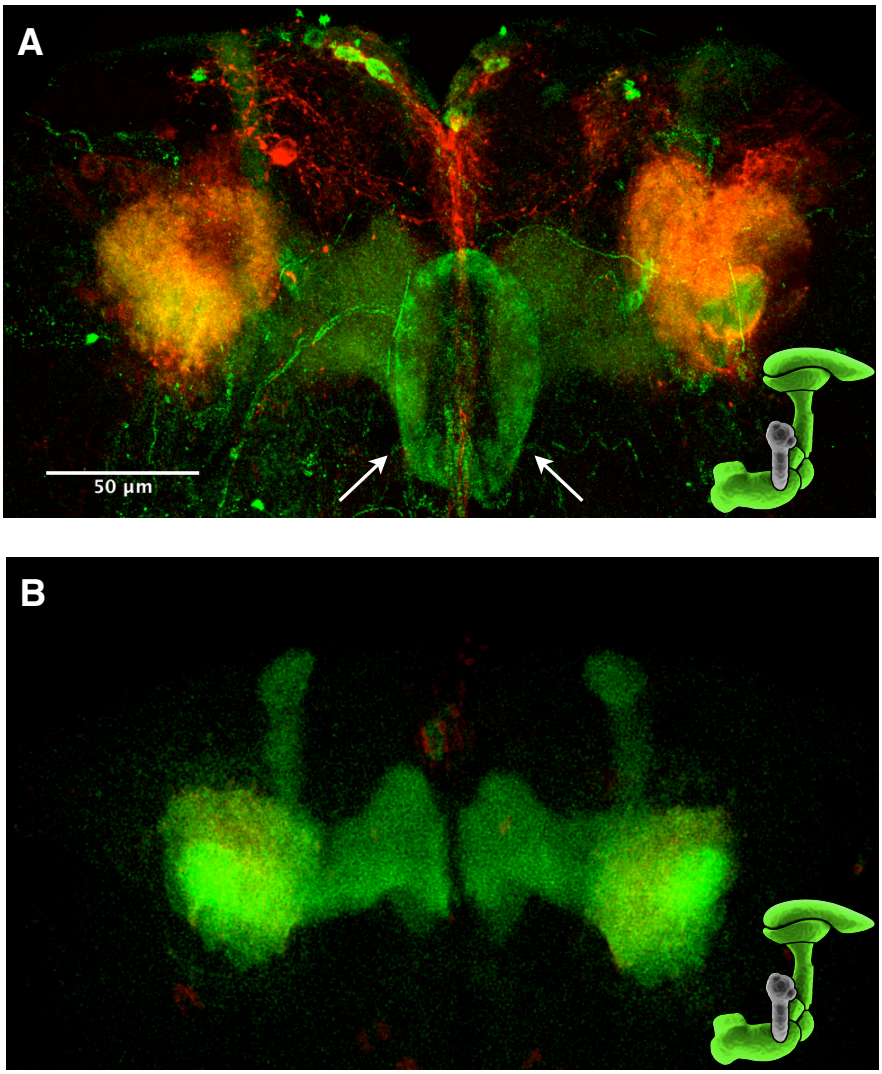


Figure 10: Localization of GFPgpi in the MBs young (A) and aged flies (B)

(A) *GFPgpi was uniformly distributed to axons, dendrites and cell body in the MBs of young flies. Additionally, GFPgpi was present in the outer ring of the ellipsoid bodies, indicated by the white arrows between the two MB*

hemispheres. ***(B) I****n aged flies, GFPgpi remained uniformly distributed in the MB, whereas it was absent from the ellipsoid bodies. The Genotype of the brains is 201Y Gal4;UAS-DenMark/UAS-GFPgpi.*
Schemes in the lower right corner: Preferential localization of GFPgpi is indicated in bright green.

5.3. Microtubule associated protein

Considering the organization of the microtubule cytoskeleton, neuronal polarity is reflected in the distinct localization of microtubule associated proteins (MAPs) to axons or dendrites [89]. Therefore we selected a well known MAP protein with reported axonal localization to monitor potential changes in the polarized distribution of axonal MAP.

5.3.1. Mouse Tau protein mislocalizes in *Drosophila* neurons

Tau proteins are microtubule associated proteins [90] with six identified Tau protein isoforms in mammals and one identified homolog in *Drosophila* [91,92]. Tau proteins are highly enriched in the nervous system, whereas they are less abundant in non-neuronal cells. The major function of Tau is the modulation of the stability of axonal microtubules [91,93] and activity, as well as localization of Tau proteins is regulated by phosphorylation at several residues. Dephosphorylated, microtubule binding Tau (dephospho-Tau) is not present in dendrites and is mainly active in the distal part of the axons, where it stabilizes microtubules and reduces catastrophe events in mammalian cells [91,93]. Dephospho-tau is well established axonal markers in mammalian cells and has been used for the same purpose in *Drosophila*, although controversial results have been obtained for the localization of exo- and endogenous Tau proteins. Tagged versions of mammalian Tau proteins have been proposed to preferentially localize to the axon of *Drosophila* neurons [41,49], although some reports suggest additionally a dendritic localization [40,94].

Young flies. We detected an intense signal of GFP-tagged mouse Tau (mTau-GFP) in the cell body, dendrites and proximal axons of MB neurons in young flies. Hardly any signal could be detected in distal axons, suggesting a preferential localization of mTau-GFP to the somatodendritic compartment and the proximal axon. The strong signal in proximal axons corresponds to the findings of Rolls and colleagues, who claimed that bovine Tau-GFP preferentially labels proximal axons, but the lower intensity in dendrites described by the authors could not be observed, therefore we can

not assert their findings [41]. But it has to be noted that the authors claimed in a later review an axonal and dendritic localization of bovine Tau, based on the same results [40].

Localization of mTau-GFP could not be determined in the aged brain, because its expression in the MBs led to an early decrease of fitness and a precocious mortality 20-25 days posteclosion. Considering the lethality of mTau-GFP expression, it is doubtful that the observation of mTau localization in young flies represents the distribution of endogenous *Drosophila* Tau protein.

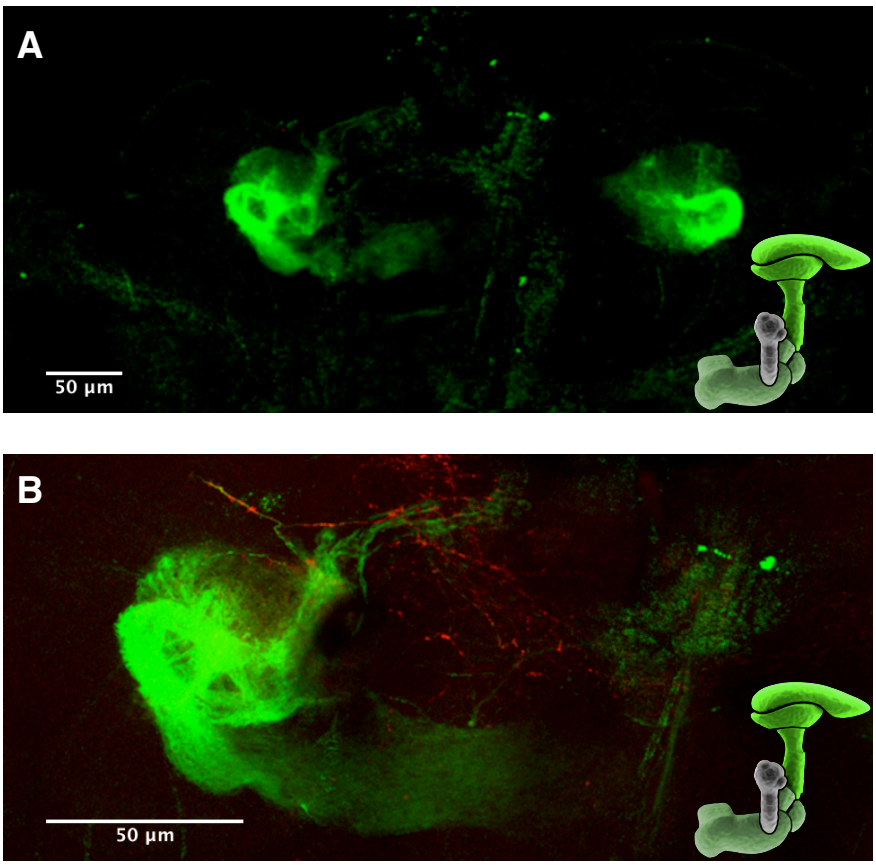


Figure 11: Localization of mTau-GFP in the MBs young flies

(A) *20x overview of mTau-GFP localization in the MBs of young flies. mTau-GFP preferentially localized to the somatodendritic compartment, whereas it only minor localized to distal parts of the axons.* ***(B)*** *Close up view of the left MB with 60x objective. The Genotype of the brains is 201Y Gal4;UAS-DenMark/UAS-mTau-GFP.*
Schemes in the lower right corner: Preferential localization of mTauGFP is indicated in bright green and minor localization in faint green.

5.4. *Motor proteins and adaptors*

Microtubule (MT)-binding motor proteins and associated adapter proteins are essential for the generation and maintenance of neuronal polarity, as they provide the key

mechanism for polarized trafficking of proteins and other subcellular components required for axonal and dendritic function. A plethora of motor proteins and associated adaptors for anterograde axonal transport has been identified, which were shown to selectively localize to the axonal compartment [95-99]. To determine whether motor proteins and adaptors preferentially localize to the axon of *Drosophila* MB neurons and if this distribution is maintained with age, we monitored the distribution of an exemplary GFP-tagged motor and adaptor protein for anterograde axonal transport in young and aged flies. Attempts have been made to monitor the localization of kinesin heavy chain (khc), a core component of kinesin 1, the primary motor for anterograde axonal transport [100]. But in accordance to previous reports regarding its expression with a pan-neuronal driver [Cook and Estes, 2006, Personal Flybase communication], expression of Khc-eGFP in the MBs was lethal and we could not assess the localization of Khc-eGFP in the MBs.

5.4.1. Anterograde microtubule-binding motor protein Immaculate connections maintains its axonal localization with age

Immaculate connections (Imac; FlyBase name unc-104) is a recently discovered member of the kinesin type 3 family, which shows significant homology to kinesin type 3 family members in other species, such as Unc-104 in Caenorhabditis elegans and Kif1a and Kif1bβ in mice [101,102]. Imac is a highly specific motor protein, selectively required for the anterograde transport of presynaptic components to the axon [101,102]. This squares with the previously reported localization of endogenous Imac and Imac-GFP to the axon of *Drosophila* motor neurons and near synapse rich regions of the brain [101,102].

Young flies. In the young stage, Imac-GFP was detected exclusively in the main axonal tracts, corresponding to the peduncle and the lobes (Figure 12A). Imac-GFP signal was specifically enriched in axon terminals, corresponding to the bulbs of the MB lobes and decreased in the axons with proximity to the somatodendritic compartment. Moreover it was slightly enriched in the heel region, which has previously been reported to harbor synaptic contact sites of MB neurons with extrinsic neurons [62,103]. Our observations indicate that Imac-GFP preferentially localizes to the axon in the MBs of young flies with an enrichment in axonal terminals and correspond to the previously reported localization of endogenous Imac and Imac-GFP to the axon

of *Drosophila* motor neurons and near presumably synapse rich regions of the brain [101,102].

Aged flies. In comparison to the young stage, a general enrichment of Imac-GFP in the distal proportion of the axons was observed. Fluorescence in the proximal axons (peduncle) had decreased compared to young flies and the enrichment specifically in the terminals of the axons was more pronounced. A negligible signal in the dendrites was detected, which can be contributed to the persistent Gal4-driven expression of Imac-GFP and thus excessive protein levels. Overall, no substantial alterations in the distribution of Imac-GFP were observed in aged flies (Figure 12B), suggesting that there are no alterations in the polarized behavior of this protein.

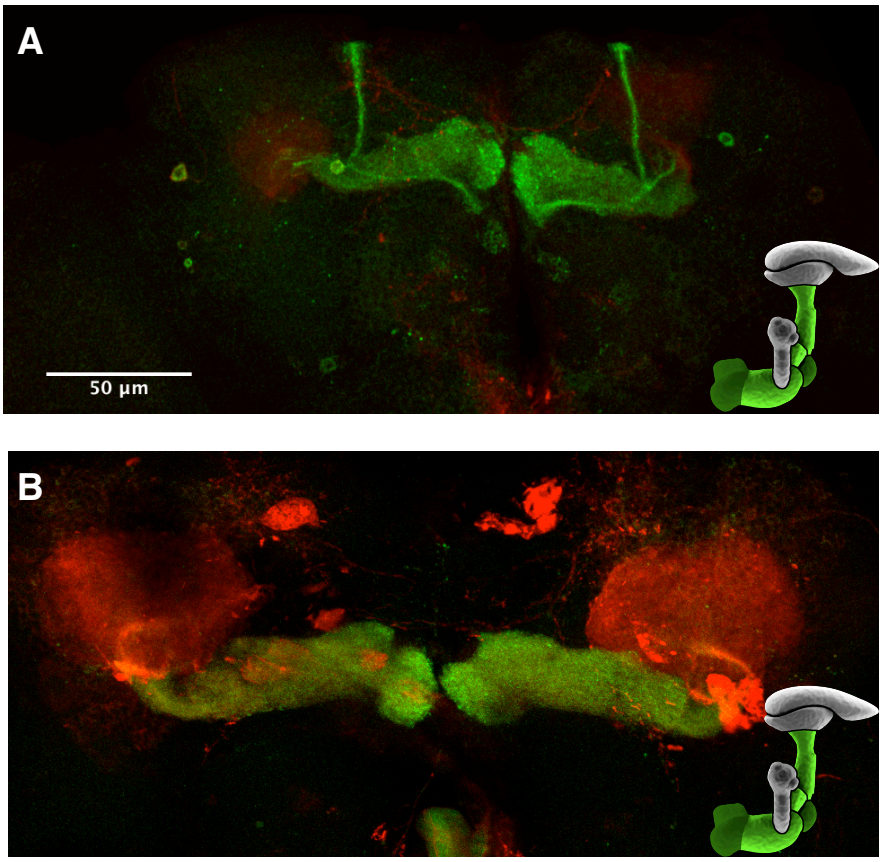


Figure 12: Localization of Imac-GFP in the MBs young (A) and aged flies (B)

(A) *In young flies, Imac-GFP selectively localized to axons with an enrichment in axon terminals of the bulb and the heel region.* ***(B)*** *Imac-GFP maintained its preferentially axonal localization and remained enriched in the bulb and at the heel region. The Genotype of the brains is 201Y Gal4;UAS-DenMark/UAS-Imac-GFP.*

Schemes in the lower right corner: Preferential localization of Imac-GFP to the axonal compartment is indicated in bright green and concentration in the bulbs and at the heel are shown in dark green.

5.4.2. Motor protein associated adapter protein APP-like-protein-interacting-protein I remains preferentially localized to axonal terminals with age

App-like-protein interacting-protein 1 (Aplip1) is a neuronally expressed *Drosophila* homolog of the mammalian Janus Kinase (JNK) scaffolding protein 1, JIP1 [97]. JIP 1 and its homologs have been shown to act as physical linkers between Kinesin 1 and different types of anterograde transported vesicles, including App (or App-like protein in *Drosophila*) or synaptobrevin containing vesicles in the axon of cultured neurons [104,105]. Furthermore, Aplip1 has been implicated to be involved in the regulation of retrograde mitochondrial transport [97]. Jip1, the mouse homolog of Aplip1, has been shown to localize to the axonal compartment [106] and transgenic Aplip1 proteins seem to primarily localize to the axon of cultured *Drosophila* neurons [97].

Young flies. Aplip1-eGFP was selectively detected in the most distal part of the axon, that is the tips or bulbs of the lobes (Figure 13A). Aplip1-eGFP signal was absent from all other subcellular compartments of the MBs. In consistency with previous reports [97], the highly distinct signal of Aplip1-eGFP in the distal part of MB axons suggests that Aplip1-eGFP selectively localizes to the most distal part of the axons, corresponding to axon terminals.

Aged flies. Aplip1-eGFP signal remained highly selectively localized to axon terminals in the MB lobes, although the signal appeared slightly more dispersed over the distal shaft of the axons, corresponding to the lobes (Figure 13B). Overall, no significant changes in the distribution of Aplip1-eGFP with age were observed, suggesting that the distinct localization of Aplip1-eGFP is maintained with age.

Overexpression of the same UAS-insertion in motor neurons was reported to cause 100% lethality during late larval and pupal development [97] and expression of Aplip1-eGFP in a different type of neurons (LNv neurons, results not shown) lead to a complete disruption and degeneration of these neurons in the adult. No such degenerated phenotype was observed with the 201Y driver. This might indicate that 201Y-driven expression is less strong or that 201Y neurons are less susceptible to the overexpression of Aplip1-eGFP.

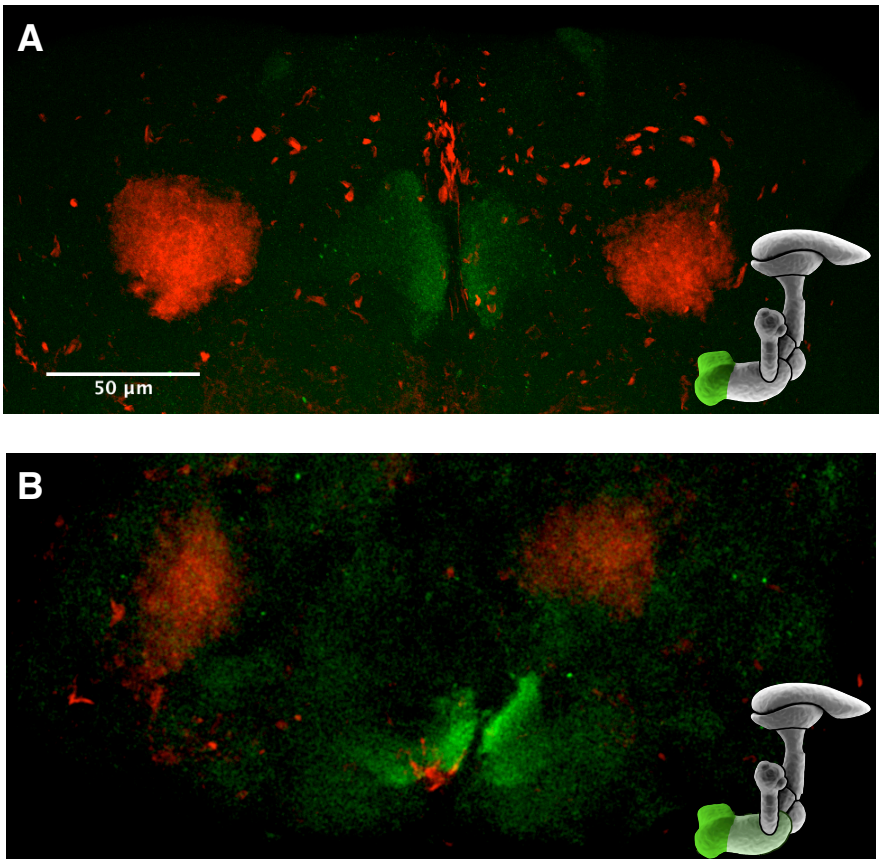


Figure 13: Localization of Aplip1-eGFP in the MBs young (A) and aged flies (B)

(A) *Aplip1-eGFP specifically localized to the bulbs of the lobes in the MBs of young flies.* ***(B)*** *In aged individuals, Aplip1-eGFP remained highly concentrated in the bulb, but appeared slightly more dispersed over the shaft of the lobes. The Genotype of the brains is 201Y Gal4;UAS-DenMark/UAS-Aplip1-eGFP.*

Schemes in the lower right corner: Preferential localization of Aplip-eGFP is indicated in bright green and minor localization in bright green.

5.5. Presynaptic proteins

An important characteristic of neuronal polarization is the differential localization of synaptic constituents to axonal or somatodendritic domains [2,40,107,108]. Presynaptic proteins, such as voltage gated ion channels, scaffolding proteins and synaptic vesicle proteins are selectively targeted to the axonal compartment and cluster in specialized axonal microdomains, forming the presynapse, respectively active zone [2,107-109]. The polarized distribution of presynaptic proteins is crucial for the functional integrity of synapses and of irreplaceable importance for neuronal function. Thus, we examined the polarized distribution of presynaptic proteins in the MBs and its maintenance with age by virtue of monitoring the distribution of fluorescently

tagged integral presynaptic active zone proteins and synaptic vesicle associated proteins in young and aged flies.

5.5.1. Active zone Proteins

5.5.1.1. Bruchpilot localizes to the cell body and dendrites and becomes enriched in axons with age

Bruchpilot (Brp), german for „crash pilot“ is a member of the conserved CAST (CAZ-associated structural protein)/ERC (ELKS Rab3- interacting protein CAST) protein family [110]. Like most members of the CAST/ERC family. Brp is a structural protein that localizes to the active zone (AZ) of the presynapse [109-111] and was shown to be a direct, but not integral component of electron dense centers of the active zone, so called T-bars [109]. Brp is thought to act as „gatekeeper“, for the assembly and organization of the active zone, where it facilitates the clustering of voltage gated calcium channels (Ca^{2+}-channels), by interaction with the α1 subunit of the voltage gated Ca^{2+}-channels, Cacophony [109-112]. It is present at all synapses in the nervous system of *Drosophila* and frequently used as a presynaptic marker for cytological studies.

Young flies. Brp-GFP was strongly expressed in the MBs of young flies, intensively labeling cell bodies, dendrites and axons. The strongest signal was detected in the cell bodies, where Brp-GFP appeared to accumulate in the cytoplasm or nucleus, as the signal pattern was different from membrane proteins, such as mCD8-GFP (Figure 6A and B). Brp-GFP was strongly detected in the center of the dendrites, but less abandoned in distal arbors. Apart from the strong signal in the cell bodies and central dendrites, Brp-GFP strongly labelled axons and was present all over the length of the axonal shaft. This result is surprising, as the MB lobes are considered the major output site of MB neurons [103,113] and hence, should majorly harbor presynaptic sites. The inordinate staining of the cell bodies may indicate an aberrant accumulation of Brp-GFP. However, our observations in the MBs of young flies indicate no preferential localization of Brp-GFP to either axons or dendrites. Brp-GFP appears to localize to both compartments and massively accumulates in the cell bodies (Figure 14A). It should be noted that discrete fluorescent signals were observed in other parts of the brain, appearing as dispersed, strongly fluorescing dots all over the brain, which re-

sembled the signal in the cell body area of the MBs and indicate a leaky expression of the UAS-insertion in extrinsic brain structures.

Aged flies. Brp-GFP remained to be strongly detected in the cell bodies and axonal tracts, whereas it was depleted from dendrites (Figure 14B). Compared to the young stage, Brp-GFP signal in cell bodies and dendrites was remarkably reduced, while it increased in the axons. A remote redistributed in the axon was observed, as Brp-GFP strongly labelled distal segments of the axons in the lobes, while being almost completely absent from proximal segments of the axons. Nevertheless, no preferential localization to axonal terminals in the tips of the lobes could be observed. In summary, Brp-GFP appeared to preferentially localize to more distal parts of the axons in the MB of aged flies, but remained to be present in the cell bodies. The increase in axons compared to dendrites suggests age-dependent changes in the distribution of Brp-GFP, manifested by an enrichment in the axonal compartment. Whether this can be considered an intrinsic property of Brp protein in the MBs is questionable, as excessive levels of Brp-GFP might have caused a mislocalization or aggregation of the protein in the cell bodies and adjacent dendrites, most pronounced in young flies.

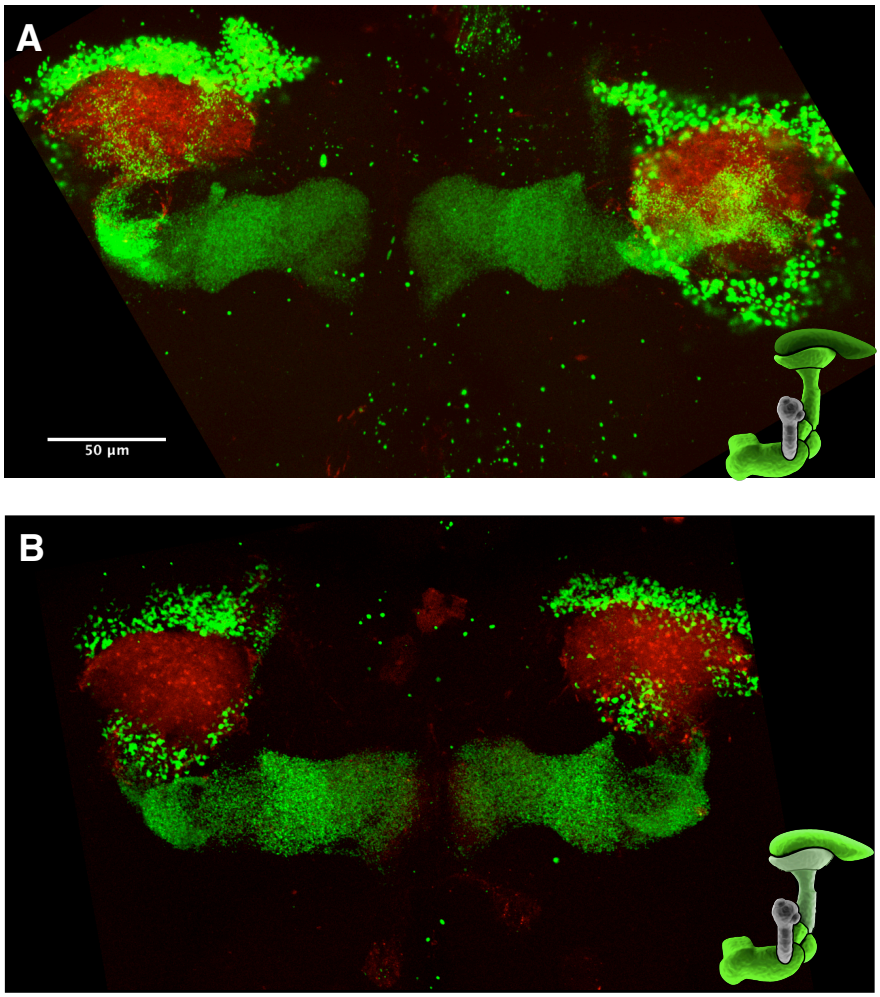


Figure 14: Localization of Brp-GFP in the MBs young (A) and aged flies (B)

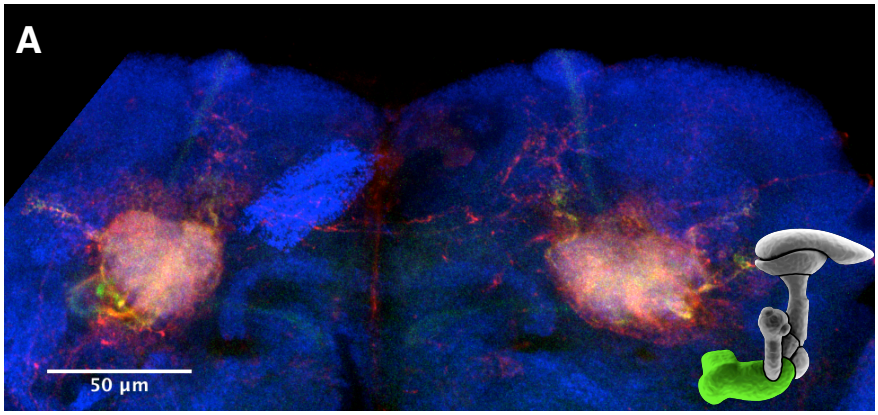
(A) *Brp-GFP massively accumulated in the cell bodies and was equally distributed to the center of the dendrites and axons in the MBs of young flies.* ***(B)*** *Brp-GFP preferentially localized to distal axons within the MB lobes in aged flies. It is depleted from dendrites and notably reduced in the cell bodies and proximal parts of the axons. The Genotype of the brains is 201Y Gal4;UAS-DenMark/UAS-Brp-GFP.*

Schemes in the lower right corner: Preferential localization of Brp-GFP is indicated in bright green. Increased concentrations of Brp-GFP are shown in dark green and minor localization in bright green.

5.5.1.2. Endogenous Bruchpilot localizes to distal axons in young flies

In order to determine whether the above described detection of Brp-GFP in dendrites and cell bodies of MB neurons reflects the localization of endogenous Brp, we stained brains of young flies with Brp antibodies. Additionally we expressed mCD8-GFP and DenMark to trace the shape of the MBs and determine the subcellular localization of endogenous Brp.

Young flies. In contrast to Brp-GFP, no endogenous Brp was present in the cell bodies of MB neurons in young flies. In the dendrites, a dense pattern of discrete fluorescent dots was present, which most likely correspond to the large synaptic buttons of extrinsic projection neurons (PN), which form extensive synaptic connections with the dendrites of MB neurons in the calyx. Brp staining did not appear to colocalize with DenMark nor mCD8-GFP in the MB dendrites (Figure 15 D-G). At the base of the dendrites, in the transition to the axons, Brp staining appeared to colocalized with both mCD8-GFP and DenMark. The compartmental identity of this segment is difficult to assess, but has been proposed to harbor the axon initial segment [41]. No Brp was detected in proximal axons of the peduncle, but homogeneously labelled the distal shaft of the axons from the heel region on (Figure 15 A-C). A slight concentration of the signal was observed in the tips of α and a‘ lobes, suggesting an concentration of Brp in axon terminals of α and α‘ lobe neurons. Localization to the tips of γ or β lobe could not be determined, as the signal was partially superimposed by signals from overlying brain structures. Taken together, endogenous Brp appeared to localize preferentially to distal parts of the axon in young flies. It was absent from the cell body and presumably dendrites, suggesting that the localization of Brp-GFP to these compartments in the MBs of young flies was an artifact of Gal4-driven expression. Except for the accumulation of Brp-GFP in the cell bodies, the distribution of endogenous Brp majorly resembled the distribution of Brp-GFP in aged flies.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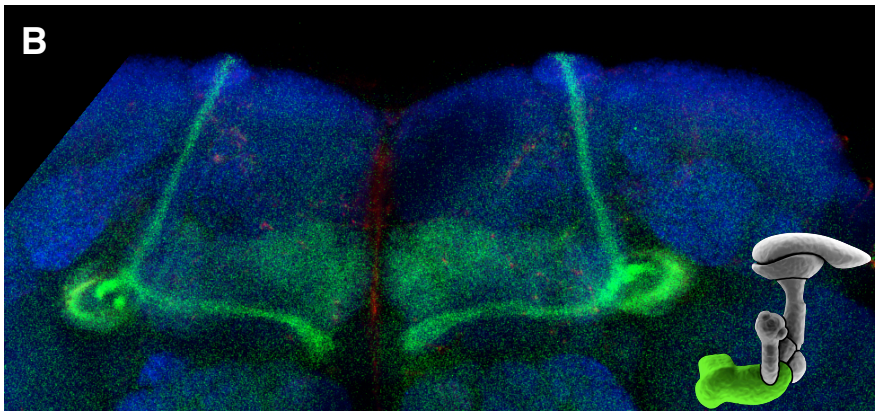
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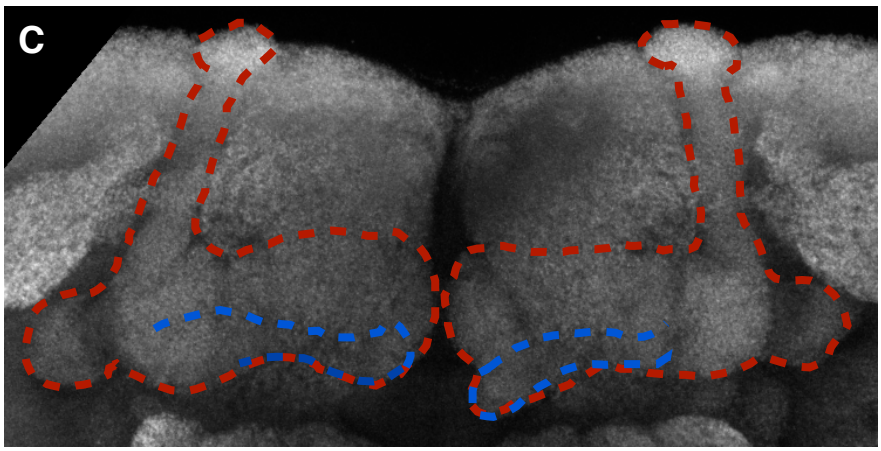
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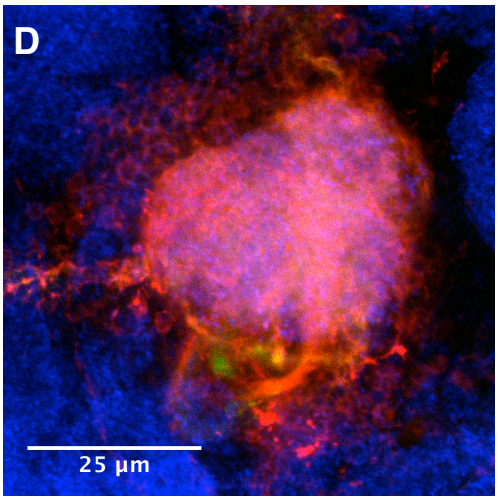
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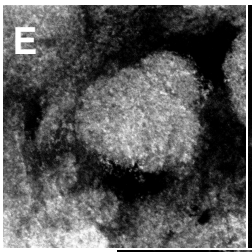
25 µm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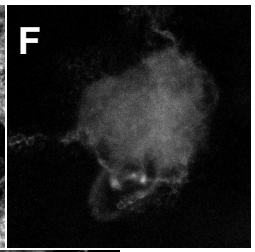
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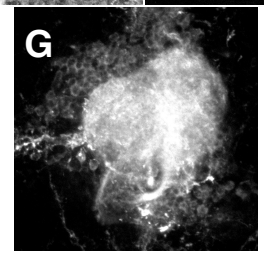
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Figure 15: Localization of endogenous Brp in the MBs young flies

(A) *Composite confocal image of of the paired MBs, stained with Brp-antibody nc82 (blue), DenMark (red) and mCD-8-GFP 8 (green).* ***(B)*** *Partial composite confocal image of the MB lobes. mCD8-GFP (green) traces the shape of the MBs and colocalized with Brp in the MB lobes.* ***(C)*** *Single channel confocal image of Brp (blue) staining in the MB lobes. The shape of the horizontal α/α' and the vertikal γ lobe is outlined in red and β/β' lobe in blue. Brp was abandoned over the whole shaft of the MB lobes and was concentrated in the bulbs of the α/α ' lobes, corresponding to axon terminals. Images A-C were obtained with an 40x oil objective. Schemes in the lower right corner: Preferential localization of endogenous Brp-GFP is indicated in bright green. For consistency the localization to the α/α' lobe is not indicated, as we based our observations of the localization of candidate proteins on the γ lobe.* ***(D)*** *Composite confocal image of the MB calyx, stained with Brp-antibody nc82 (blue), Denmark (red) and mCD-8-GFP 8 (green) revealed no colocalization of Brp with mCD8-GFP or DenMArk in the calyx.* ***(E-G)*** *Single channel composite confocal images of Brp (blue, B), mCD8-GFP (green, C) and DenMark (red, D). The intense punctuated pattern of Brp in the dendritic MB calyx most likely corresponds to the large synaptic boutons of extrinsic PNs. Images were obtained with an 60x objective and 3-fold zoom. The Genotype of the brain is 201Y Gal4;UAS-DenMark/UAS-mCD8-GFP.*

5.5.1.3. Bruchpilot interaction partner Cacophony is homogeneously distributed in young and becomes axonally enriched with aged

Cacophony (Cac) gene encodes α1 subunit of presynaptic voltage gated Ca^{2+}-channels [114] and is the only homolog of vertebrate α1 subunits of presynaptic N and P/Q type voltage gated Ca^{2+}-channels in the *Drosophila* genome [115]. All voltage gated Ca^{2+}-channels in *Drosophila* are composed of one α-subunit (Cac), auxiliary β and α2d subunits [115].These voltage gated Ca^{2+}-channels are crucial for chemical synaptic transmission and mediate calcium influx required for evoked neurotransmitter release [116-118]. Endogenous Cac and a transgenic UAS-construct composed of Cac fused to eGFP were shown to localize to the presynaptic active zone at the larval neuromuscular junction [119-121].

Young flies. Cac-eGFP was strongly detected in the cell bodies and rather homogeneously distributed to dendrites and axonal tracts (Figure 16 A). The strong signal in the cell bodies indicates, a strong expression of Cac-eGFP in the MBs, which may have resulted in an aberrant accumulation of the protein in the plasma membrane of the cell bodies, as observed for Brp-GFP. Axonal fluorescence of Cac-eGFP appeared to be slightly stronger in proximal axons of the peduncle than in distal axons of the lobe, but the difference can be contributed to the highly concentrated array of axons within this region. In sum, Cac-eGFP preferentially localized to the cell body of

young MB neurons and was homogeneously distributed to axons and dendrites. This distribution resembles the distribution of Brp-GFP in young flies, which is not surprising as Brp and Cac were shown to directly interact and colocalize at the neuromuscular junction [111]. However, the observed distribution of both proteins in the MBs does not resemble the localization in *Drosophila* motor neurons, where both presynaptic proteins localize to the axon and cluster at presynaptic sites in axon terminals [111,119]. But as we observed notable differences in the distribution of Brp-GFP compared to the endogenous protein, it is likely that the distribution of Cac-eGFP may not reflect the distribution of the endogenous protein in young flies.

Aged flies. In contrast to young flies, Cac-eGFP signal was preferentially detected in the axon of MB neurons, whereas the signal in the cell body and dendrites was diminished. In the axons, Cac-eGFP appeared to be uniformly distributed along the whole length of the axonal shaft. As for Brp-GFP, no enrichment of this presynaptic protein could be observed in axon terminals of the lobes. Considering that the accumulation of Cac-eGFP was a result of excessive Gal4-driven expression, it can be assumed that Cac-eGFP preferentially localizes to the axons of MB neurons in aged individuals. Thus, the distribution of Cac-eGFP changed in dependence of age, from a rather homogeneous distribution in the young to a preferentially axonal localization in the old. These results resemble the age-dependent alterations in the distribution of its interaction partner Brp-GFP. Then again, it is disputable to which extent these alterations represent the condition of the endogenous protein.

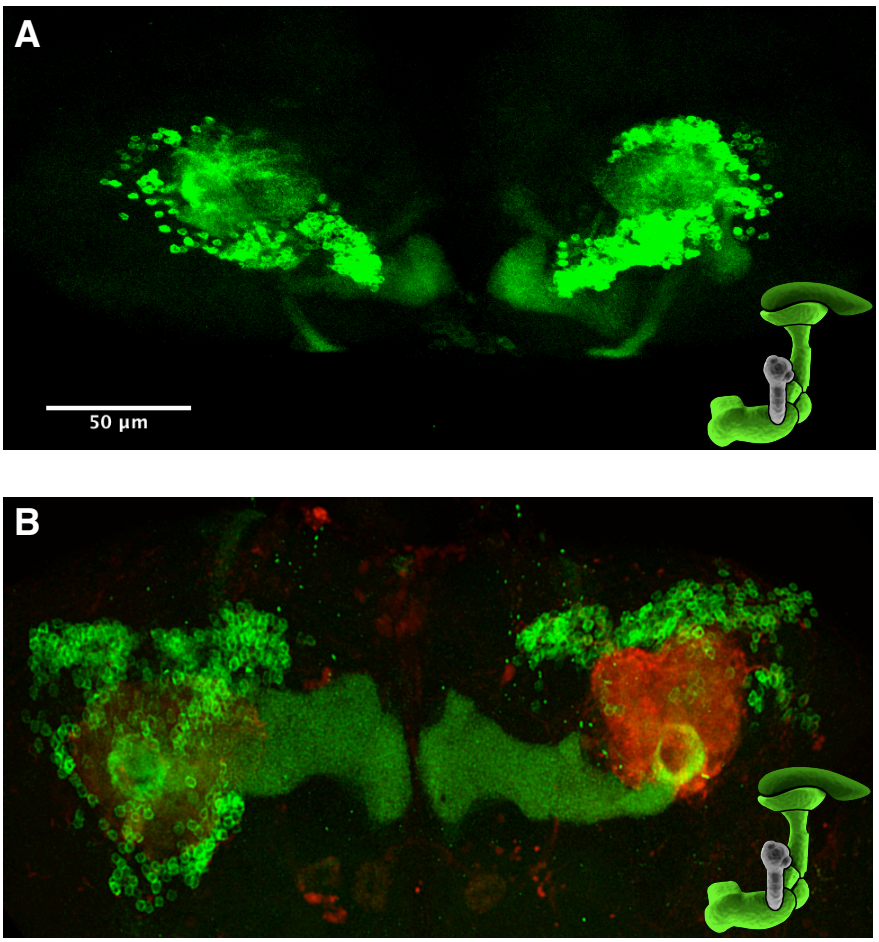

Figure 16: Localization of Cac-eGFP in the MBs young (A) and aged flies (B)

(A) *In MB neurons of young flies Cac-eGFP, was highly concentrated in the plasma membrane of the cell bodies- It was less abandoned in neuronal processes and uniformly distributed to axons and dendrites.****(B)*** *Cac-eGFP preferentially localized to the axons of the MBs in aged flies. Compared to young flies, it was notably reduced in the cell bodies and dendrites. The Genotype of the brains is 201Y Gal4;UAS-DenMark/UAS-Cac-eGFP.*
Schemes in the lower right corner: Preferential localization of Cac-eGFP is indicated in bright green. Increased concentrations are shown in dark green and minor localization in bright green.

5.5.2. Synaptic vesicle-associated proteins

5.5.2.1. Synaptotagmin 1 preferentially localizes to distal axons in young and becomes enriched in dendrites and proximal axons with age

Synaptotagmin 1(Syt1) is a member of the widely conserved protein family of Synaptotagmins, with seven identified family members in *Drosophila* and 19 in mammals [122]. Syt1 represents the most abundant Ca^{2+}-binding protein present on synaptic

vesicles and is considered the major low affinity Ca^{2+}-sensor for synchronous synaptic vesicle exocytosis, required for efficient synaptic transmission [122,123]. Additionally, a potential role of Syt1 in vesicle recycling has been proposed [122]. Endogenous Syt1 was shown to accumulate near predicted synapse rich regions in adult flies and third instar larvae [124,125]. Due to its high abundance on synaptic vesicles, Syt1 is frequently used as a marker for synaptic vesicles or axon terminals.

Young flies. Syt1-eGFP appeared to be highly expressed in the MBs and was preferentially detected in the major axonal tracts, where it was enriched in distal parts of the axons (Figure 17A). Hardly any Syt1-eGFP was detected in the cell bodies and only a minor fluorescence was present in the dendritic calyx. Enrichment in distal axons started at the heel region, where Syt1-eGFP was specifically enriched. It strongly labelled the shaft of the lobes and again increased in axon terminals within the bulbs of the lobes, where the highest intensity of Syt1-eGFP was detected. This observation is consistent with the observed distribution of endogenous presynaptic marker Brp, suggesting a proper localization of Syt1-GFP to synaptic vesicles and enrichment near presumably presynaptic sites in distal axons [59,63,64]. Leaky expression of Syt1-eGFP was observed in extrinsic neurons that project to the MB calyx, the projection neurons (PN). In PN, Syt1-eGFP exclusively localized to axon terminals of presumably presynaptic specialization, since PNs are considered to provide major input to the MB calyx. The observation that Syt1-eGFP selectively localized to axon terminals in PN could further indicate that excessive Gal4-driven expression levels in the MB indeed caused a minor mislocalization of Syt1-eGFP to dendrites. Else, it may suggest the presence of non-classical synaptic connections in the MB calyx, as proposed by Rolls et al. 2007. But our above described observation that endogenous Brp appears to be absent from dendrites contradicts the assumption of Rolls and colleagues, as Brp is considered to be an essential component of presynaptic sites in the *Drosophila* nervous system. However, our observation of Syt1-eGFP localization in the MBs indicates, that it preferentially localizes to the axon and is enriched in axonal terminals of young individuals, with a negligible localization to dendrites.

Aged flies. Compared to young flies, a general enrichment of Syt1-eGFP signal was observed in all subcellular compartments. The abundance of Syt1-eGFP signal in the dendritic region of the calyx slightly increased, but a more pronounced increase was observed in the axons. Syt1-eGFP signal was most notably increased in distal axons,

but the specific concentration in axon terminals and at the heel region is lost. Overall, Syt1-eGFP appeared to become enriched in the upper part of the MBs, meaning dendrites and proximal axons, compared to young flies. But as Syt1-eGFP signal as well increased in distal axons of the lobes, persistent Gal4-driven expression and resulting excessive protein levels are likely to account for the increase in the upper part of the MBs. Hence, it is doubtful whether the observed enrichment of Syt1-eGFP in dendrites and proximal axons represents notable changes in the distribution of endogenous Syt1 and accordingly synaptic vesicles in the MBs of aged flies. In any case, Syt1-GFP remained preferentially localized to the axonal compartment with a concentration in distal segments (Figure 17B), reflecting the previously shown concentration of Syt1 near synapse rich regions [124,125].

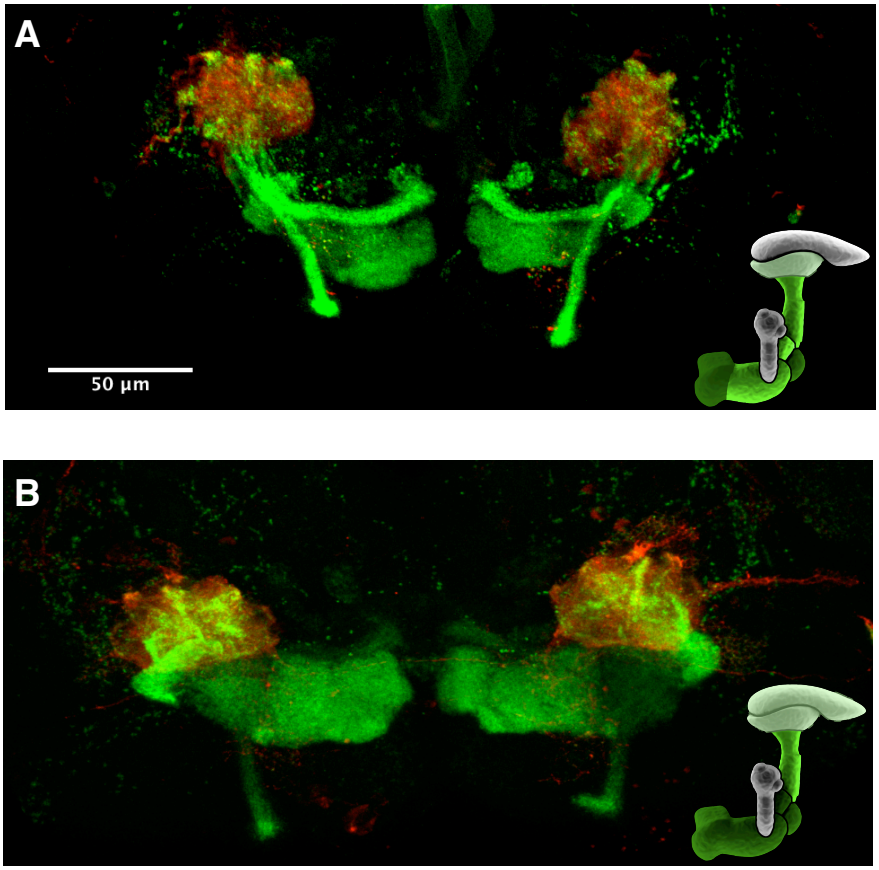

Figure 17: Localization of Syt1-eGFP in the MBs young (A) and aged flies (B)

(A) *In MB neurons of young flies Syt1-eGFP, was preferentially localized to the axonal compartment and was concentrated in the heel region and in the bulbs of the lobes. Syt1-eGFP minor localized to dendrites and was absent from cell bodies. Leaky expression of Syt1-eGFP in PN neurons and its selective localization to MB contacting axon terminals is evident to the left and right of the two MB calyxes.* ***(B)*** *In the MBs in aged flies, Syt1-eGFP remained preferentially localized to distal parts of the axons, but the concentration in the bulbs and at the*

heel region is lost. Compared to young flies, increased leakage of Syt1-eGFP into the dendrites and the cell bodies was evident. The Genotype of the brains is 201Y Gal4;UAS-DenMark/UAS-Syt1-eGFP.
Schemes in the lower right corner: Preferential localization of Syt1-eGFP is indicated in bright green. Increased concentrations are shown in dark green and minor localization in bright green.

5.5.2.2. Preferential localization of Ras-like protein in rat brain 3 to the axon is majorly maintained with age.

Ras-like protein in rat brain 3 (Rab3) belongs to the Ras superfamily of small GTPases, which are involved in various aspects of membrane trafficking [122,126,127]. One Rab3 gene has been identified in *Drosophila* [126], while 4 members of the Rab3 subfamily have been identified in mammals, namely Rab A, B, C and D [127]. Rab3 is highly expressed in neuronal cells of *Drosophila* embryos [126] and is one of the most abundant proteins associated with synaptic vesicles. The precise function of Rab3 is currently unknown, but GTP-dependent regulatory functions of Rab3 in various aspects of synaptic vesicle exocytosis have been hypothesized, including docking, priming and fusion of synaptic vesicles [122,127]. When expressed by the Gal4 system, YFP tagged Rab3 was found to localize almost exclusively to synaptic terminals of photoreceptors of 3rd instar larvae [126]. Rab3 was chosen as a candidate for its reported localization to synaptic terminals in larvae [126] and its association with synaptic vesicles, which might allow the validation of results, obtained for Syt1.

Young flies. Rab3-YFP was detected almost exclusively in the axons and only occasionally a weak signal was present in the dendrites (Figure 18A). It was relatively homogeneously distributed along axons, with a minor increase in the heel region of the terminating peduncle and the axonal terminals in the bulbs of the MB lobes. An increase in the heel region and axon terminals was observed for all proteins involved in axonal trafficking of synaptic vesicles, resp. Syt1-eGFP and Imac-eGFP. However, overall our observations suggest a highly selective localization of Rab3-YFP to the axons in young individuals, which is consistent with previous reports in larval photoreceptor cells [126] and resembles the above described distribution of Syt1-eGFP.

Aged flies. As for Syt1-eGFP, a general increase of Rab3-YFP signal was observed in all subcellular compartments in aged flies. In the axons, the increase in distal parts was more pronounced than in proximal parts, but the specific enrichment in axon terminals and at the heel region is lost. Occasionally, Rab3-YFP was weakly detected

in the cell bodies of some aged individuals, potentially associated with the plasma membrane, as it resembled the distribution of membrane markers. In comparison to young flies, a weak, but intensified signal was abandoned in the dendritic calyx. Nevertheless, Rab3-YFP remained preferentially localized to the axonal compartment with a slight increase in the somatodendritic compartment in aged individuals (Figure 18B). These minor age-dependent alterations of Rab3-YFP localization resembled alterations of Syt1-eGFP localization, but are less evident. Rab3-YFP appeared to be lower expressed than Syt1-eGFP, which might be the cause for the less pronounced increase in the somatodendritic compartment and proximal axons. As for Syt1-eGFP, the persistent expression of Rab3-YFP is likely to account for the observed increase in the somatodendritic compartment and localization of Rab3-YFP appears to be majorly maintained with age.

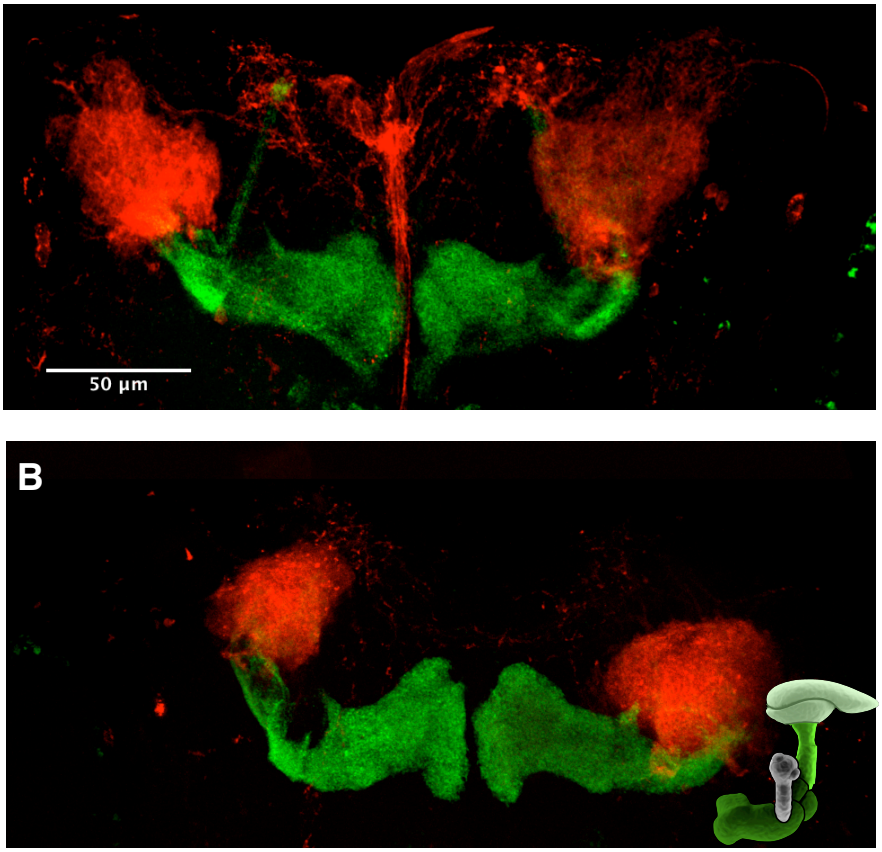

Figure 18: Localization of Rab3-YFP in the MBs young (A) and aged flies (B)

(A) *In young individuals, Rab3-YFP selectively localized to the axonal compartment and was concentrated distal parts of the axons, respectively the heel region and the bulbs of the MB lobes.* ***(B)*** *In aged flies, Rab3-YFP majorly maintained its preferentially localization to distal parts of the axons, but the distinct enrichment in the bulbs*

and at the heel region is lost. A weak signal of Rab3-YFP was present in dendrites and the cell bodies. The Genotype of the brains is 201Y Gal4;UAS-DenMark/UAS-Rab3-YFP.
Schemes in the lower right corner: Preferential localization of Rab3-YFP is indicated in bright green. Increased concentrations are shown in dark green and minor localization in bright green.

5.5.2.3. Synaptojanin (mis)localizes to the somatodendritic compartment in young and becomes axonally polarized with age.

Synaptojanin (Synj) is a phosphatidylinositol phosphatase [128], with functional and structural homologs found in humans, mouse, *C. elegans*, and lamprey [129-131]. Synj is essentially involved in clathrin mediated synaptic vesicle endocytosis [128]. In *Drosophila*, endogenous Synj was shown to specifically localize to presynaptic terminals at the neuromuscular junction and photoreceptor terminals [128]. This selective localization was confirmed for a transgenic fusion protein, consisting of Synj and eGFP (Synj-eGFP) at the neuromuscular junction of third instar larvae [132].

Young flies. Synj-eGFP appeared to be highly expressed in the MBs of young flies, as it clearly labeled cell bodies and neuronal processes, without the need for antibody stainings. The signal of Synj-eGFP was relatively homogeneously distributed to all subcellular compartments and appeared to be slightly stronger in the somatodendritic compartment than in the axon (Figure 19A). This contrasts the previously reported localization of Synj and Synj-eGFP to presynaptic terminals in other types of neurons [128,132], and may indicate that excessive expression levels of Synj-eGFP caused an aberrant localization of Synj-eGFP, as observed for the presynaptic proteins Brp-GFP and Cac-eGFP and marginally the synaptic vesicle proteins Rab3-YFP and Syt1-GFP.

Aged flies. Compared to young individuals, Synj-eGFP signal in the somatodendritic compartment considerably decreased, whereas it increased in distal parts of the axons from the heel region on. Most notably, Synj-eGFP signal was elevated in axon terminals, near presumably synapse rich regions and resembled the distribution of proteins involved in synaptic vesicle exocytosis. In summary, Synj-eGFP appeared to polarize to the axonal compartment in aged flies (Figure 19B), contrasting the homogeneous distribution in young. The striking alteration in the distribution of this exocytotic protein fits the generally observed enrichment of presynaptic proteins, such as

Brp-GFP and Cac-eGFP as well as the endocytotic synaptic vesicle proteins, Syt1-eGFP and Rab3-YFP in the axonal compartment with age.

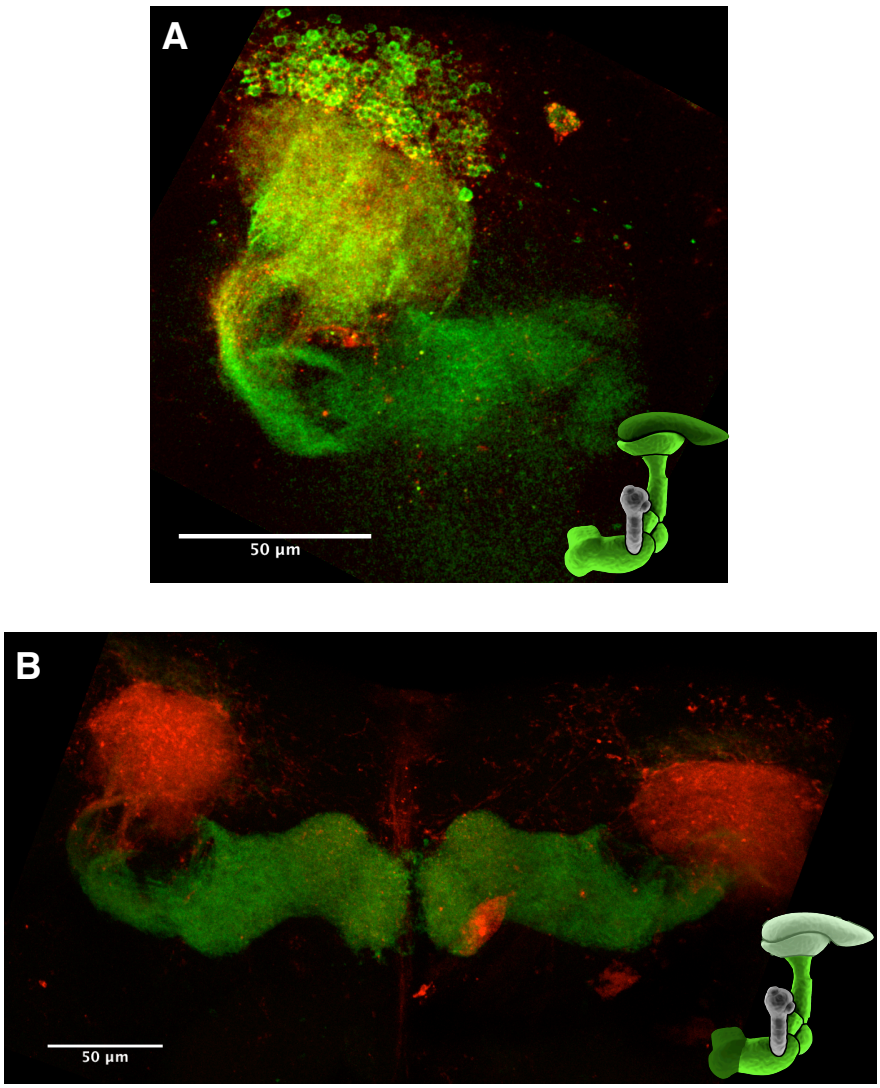

Figure 19: Localization of Synj-eGFP in the MBs young (A) and aged flies (B)

(A) *Close-up view of the left MB labelled with Synj-eGFP of a young fly, obtained with a 60x oil objective. Synj-eGFP was concentrated in the cell bodies and homogeneously labelled neuronal processes, respectively axons and dendrites* ***(B)*** *In aged flies, Synj-eGFP preferentially localized to axons with a distinct enrichment in the bulbs and was less abandoned in the somatodendritic compartment. The Genotype of the brains is 201Y Gal4;UAS-DenMark/UAS- Synj-eGFP.*

Schemes in the lower right corner: Preferential localization of Synj-eGFP is indicated in bright green. Increased concentrations are shown in dark green and minor localization in bright green.

6.Discussion

6.1. Clear polarization of *Drosophila* mushroom body neurons in young individuals

Drosophila neurons have morphologically clearly identifiable axons and dendrites, but it remains highly debated if and how *Drosophila* neurons are polarized on a molecular level. Only recently, it has become clear that many molecular aspects of neuronal polarity are conserved from *Drosophila* to mammals [40-42,48-51]. Several studies have shown the differential localization of exogenous and endogenous marker proteins to dendrites and axons in various types of neurons, including the mushroom bodies (MBs) [50,71]. Expressing transgenic GFP-fused proteins of hypothesized axonal polarization in the MBs of young flies, we could confirm the selective or preferential distribution of membrane and cytosolic proteins to the axonal compartment. Most of the examined proteins showed a differential localization to axons compared to dendrites or the cell body. Consequently, our data indicates that the unipolar neurons of the *Drosophila* MBs share major features of neuronal polarization with mammalian neurons and provide therefore a simple, genetically tractable, model to study the complex processes of neuronal polarization in vivo, such as the maintenance of neuronal polarization with age.

6.1.1. Polarization of membrane proteins

6.1.1.1. Polarization of Integral membrane proteins

In mammals, several non-synaptic membrane proteins preferentially localize to axons or dendrites. In *Drosophila*, exogenous and endogenous cell adhesion molecules have been shown to be polarily distributed to axons or dendrites in different types of neurons, including the MBs. For instance, a fusion protein consisting of the mammalian cell adhesion molecules NgCam and YFP was shown to localize to the axons of mushroom body neurons in the larval brain [41], whereas a specific splice form of GFP-tagged Dscam specifically localizes to the dendrites of MB neurons [133]. The clearest examples for axonally polarized integral membrane proteins are the roundabout receptors (Robo). GFP-fusion proteins of the three members of Robo protein family in *Drosophila*, Robo 1 to 3, were found to localize to specific segments of the

axon in cultured *Drosophila* neurons, which was confirmed in vivo, by expressing Robo-eGFP proteins in larval commisual neurons. Robo1-eGFP was found to localize specifically to the cell body and the proximal partition of the axon, whereas Robo2- and 3-eGFP primarily localized to distal axons.[71] Thus, the authors hypothesized an additional subcompartmental division of axons into distinct compartments that are characterized by the selective localization of membrane molecules involved in axon guidance and synapse formation [50,71]. In concert with these findings, we found GFP-fusion proteins of Robo 1, 2 and 3 to be preferentially localized to the axon of the MBs in 3-7 day old (young) flies (Figure 7A, 8A and 9A). GFP-tagged Robo 2 (Robo2-GFP) and Robo 3 (Robo3-GFP) selectively localized to the axons, while being depleted from the somatodendritic compartment and also GFP-tagged Robo1 (Robo1-GFP) showed a preferentially axonal localization in young individuals. Hence, it appears that *Drosophila* MB neurons share the ability to selectively target membrane proteins to certain subcellular compartments with vertebrate neurons. Moreover, we observed a selective localization to distinct segments of the axon in MB neurons for Robo2-GFP and less evident for Robo3-GFP. Both proteins were enriched in distal parts of the axons, suggesting a potential axonal subcompartmentalization of *Drosophila* MB neurons in young individuals, as it has been previously shown for larval commisual neurons [71].

6.1.1.2. Polarization of GPI-anchored

Among the first proteins identified to selectively localize to the axon in mammalian neurons were membrane associated glycosyl phospatidylinositol (GPI)-anchored proteins [85]. GPI-anchored proteins associate shortly after synthesis with glycosphingolipid rich domains in the trans-Golgi network that are thought to be involved in axonal trafficking of proteins and are subsequently targeted to the axon in primary hippocampal neurons [85,87,88]. Currently, axonal polarization of GPI-anchored proteins in *Drosophila* neurons has not been shown, but a GPI-anchored reporter protein was shown to preferentially localize to glycosphingolipid rich domains of the plasma membrane, which may have potential implications on axonal trafficking of proteins [87]. Expressing this GPI-anchored reporter protein, consisting of GFP fused to a GPI-anchor (GFPgpi) in MB neurons of young flies, we observed a uniform distribution of GFPgpi (Figure 10A), indicating no polarized distribution of GPI-anchored pro-

teins in *Drosophila* MB neurons. It has to be noted that the GFPgpi UAS insertion was expressed at basal levels in the MB in absence of the Gal4 driver. Even if expressed only at basal levels, GFPgpi was observed to be uniformly distributed, suggesting that independent of protein levels, GFPgpi is uniformly localized. Thus, it appears that GPI-anchored proteins are not targeted to the axon in *Drosophila* MB neurons, contrasting previous findings in primary hippocampal cell culture [85]. But as studies in cultured cortical neurons have proven that simple addition of a GPI-anchor is not sufficient to target exogenous proteins to the axonal compartment [134] and several GPI-anchored proteins are known to be polarily distributed to the axon in *Drosophila*, we might have to restrict our statement to the localization of GFPgpi. The uniform distribution of this transgenic protein, may not reflect a general property of GPI-anchored proteins and like in mammalian systems, simple addition of a GPI-anchor might not be sufficient to target proteins to the axonal compartment in *Drosophila*.

Apart from the targeted expression in the MB of young flies, we could additionally detect high levels of GFPgpi in the outer ring of the ellipsoid bodies, a brain structure, which forms extensive synaptic connections with the axons of MB neurons. No GFPgpi signal was present in the absence of the 201Y Gal4 driver, suggesting that the detected signal had not been caused by basal expression of GFPgpi in the ellipsoid bodies. In larval cells of the imaginal disc, GFPgpi was shown to be released from the basolateral membrane of expressing cells within membrane exovesicles, termed argosomes [135]. GFPgpi carrying argosomes travel through the intracellular space and are subsequently inserted in the apical membrane of non-expressing cells [135]. The same may apply to MB neurons, where GFPgpi is released from the plasma membrane of expressing cells and associates with the membrane of proximal, non-expressing cells, in this case the ellipsoid bodies. In addition our observation may suggest that high levels GFPgpi are required for the release from the plasma membrane, as no GFPgpi signal was detected in the outer ring of the ellipsoid bodies in the absence of Gal4, when GFPgpi was expressed at basal levels in the MBs. Argosomes have been proposed to be important for the spreading of morphogens, such as the GPI-anchored morphogen wingless [135]. The absence of GFPgpi in the ellipsoid bodies in the aged brains, might potentially suggest that this ability of spreading is lost with age, reinforcing a potential role in the young or devel-

oping brain, as MB development is thought to continue through the first week posteclosion [55].

6.1.2. Polarization of microtubule associated proteins

A key feature of neuronal polarity in vertebrates is the distinct organization of the cytoskeleton, including the differential distribution of microtubule associated proteins (MAPs). MAPs are polarily distributed in mammalian neurons and frequently used as axonal or dendritic markers [89,91]. Dephosphorylated Tau exclusively localizes to the axon of mammalian neurons [15] and is a classically used axonal marker. Although controversial results have been obtained for the localization of Tau proteins in *Drosophila*, endogenous and exogenous Tau proteins have been used as axonal markers in *Drosophila* neurons. Exogenous bovine Tau was reported to preferentially localize to the proximal axons of MB neurons and was less abundant in dendrites [41]. In a later review the authors stated a dendritic and axonal localization based on the same results, reflecting the localization of endogenous GFP-tagged Tau to dendrites as well as axons [40]. We found exogenous mouse Tau tagged with GFP (mTau-GFP) to preferentially localize to the cell bodies, dendrites and proximal axons in the MBs of young flies, with no significant enrichment in the axonal compartment. Furthermore we could observe a premature decline in fitness and early death of mTau-GFP expressing flies. This fits to a recent report in which the expression of human Tau proteins was shown to cause an accumulation of hyperphosphorylated human Tau in the somatodendritic compartment [136]. Hyperphosphorylated human Tau was mostly recovered in its soluble cytosolic species as a consequence of the poor ability of human Tau proteins to bind microtubules in *Drosophila*. Thus the localization of human Tau protein does not reflect the localization of endogenous *Drosophila* Tau [136]. The disability of human Tau to bind microtubules in *Drosophila* might as well apply to other mammalian Tau proteins and account for the high levels of mTau-GFP in the cell body and dendrites, which may have spread to the proximal axon. This would additionally explain the observed premature decline of fitness and early death of mTau-GFP expressing flies, as expression of human Tau in *Drosophila* was hypothesized to mimic the pathology of tauopathies, where accumulation of Tau proteins leads to neurodegeneration, cognitive decline and subsequently early death of the individual [136]. Thus, we conclude that available constructs of exogenous

mammalian Tau proteins can not be used as axonal markers in *Drosophila* and do not reflect the localization of endogenous Tau protein. This renders it impossible, to draw conclusions on the polarized distribution of Tau protein based on the localization of exogenous mouse Tau in *Drosophila*.

6.1.3. Polarization of anterograde motor proteins and adaptors

Motor proteins and their associated adapters are of central importance for polarized trafficking of proteins and consequently the establishment and maintenance of axonal function. Both types of transport proteins are likely to govern the specificity of neuronal transport and several were shown to selectively localize to either dendrites or axons in *Drosophila* and mammalian neurons [95-99]. For instance, a fusion protein, consisting of the motor domain of the kinesin family member nod and YFP, selectively localizes to dendrites in larval MBs [41], whereas Kinesin type 3 immaculate connections (Imac) was reported to localize to the axon of motor neurons [101]. We found two independently acting GFP-tagged proteins that are majorly involved in anterograde transport and its regulation, immaculate connections (Imac-GFP) and App-like-protein interacting protein 1 (Aplip1-eGFP) to selectively localize to the axonal compartment in the MBs of young flies. Both proteins were absent from dendrites, revealing a highly selective trafficking of these proteins to the axonal compartment. Moreover, Imac and Aplip1 were observed to be enriched in the most distal parts of the axons, which presumably correspond to axon terminals and are thought to harbor presynaptic structures [103,113]. Our observations are consistent with the previously reported localization of Imac and Aplip1 to the axon of *Drosophila* motor neurons, and the enrichment of Imac in axon terminals and near synapse rich regions of the brain [97,101,102]. Both proteins are essential for the delivery of presynaptic constituents, such as Synaptobrevin and Synaptotagmin bearing vesicles, and their preferential localization to distal segments of the axons might substantiate the presence of presynaptic sites exclusively in axon terminals within the MB lobes. Additionally we found both proteins to be enriched at the heel region, where the axons that run together through the peduncle split and innervate the different lobes. Similarly, interaction partners of Imac that are involved in the trafficking of synaptic vesicles, Syt1-eGFP and Rab-YFP, accumulated in the same region, suggesting that the heel region might constitute a bottleneck, where trafficking proteins and vesicles stall, before they

are further transported to distal parts of the axon. Furthermore synaptic connections in this region have been reported [62,103], which might further contribute for this concentration of proteins involved in axonal transport of synaptic constituents.
Our findings confirm the polarized distribution of proteins involved in anterograde transport in *Drosophila* MB neurons and highlight the specificity of motor and adaptor protein trafficking to certain subcellular compartments, which might reinforce the hypothesized function of motor proteins and adaptors in regulating the specificity of microtubule based transport to the axon.

6.1.4. Polarization of presynaptic proteins (?)

In mammalian motor or hippocampal neurons, presynaptic components are classically restricted to the axon and postsynaptic constituents to dendrites [2,107,108]. But in some mammalian interneurons, synaptic vesicles localize to dendrites and harbor presynaptic sites [69]. In *Drosophila*, the polarized distribution of presynaptic proteins has been most thoroughly studied in motor neurons, where antibody stainings and tagged proteins showed a similar distribution of pre- and postsynaptic markers as in mammalian motor neurons [40]. For example, a highly selective localization to axon terminals was observed for active zone proteins Cacophony [119-121] and Bruchpilot [110,111], as well as synaptic vesicle proteins Synaptobrevin [137] and Synaptotagmin [49,126]. Similar results have been obtained for other types of *Drosophila* neurons, including CCAP peptidergic neurons and sensory neurons [40,138,139]. But as in mammals, there are exceptions from this classic model. Central neurons that process information frequently have processes that contain both, pre- and postsynaptic structures, as exemplified by serotonergic interneurons [140]. Likewise, a dendritic localization of synaptic vesicles has been shown in MB neurons and the presence of presynaptic sites in the dendrites of MB neurons has been hypothesized [41]. In contrast, extensive immuno electronmicroscopy studies of synaptic connections in the dendritic calyx revealed no presynaptic connections in MB neurons [56,141]. Thus it remains to be elucidated wether presynaptic structures are present in the dendrites of MB neurons.
We obtained divergent results for the localization of fluorescently-tagged presynaptic proteins. GFP-tagged active zone proteins Bruchpilot (Brp-GFP) and Cacophony (Cac-eGFP) and the synaptic vesicle associated protein Synaptojanin (Synj-eGFP)

massively accumulated in the cell bodies and localized to axons as well as dendrites of MB neurons in young individuals. Subsequent antibody stainings of endogenous Bruchpilot (Brp) revealed a preferential localization of Brp to distal axons of the lobes and showed that the accumulation in the cell bodies and proximal axons of the MBs in young individuals was most likely an artifact of Gal4-driven expression, as none of the endogenous protein was abandoned in these regions. Interpretation of the antibody staining in the complex dendritic calyx was difficult, since accurate resolution of presynaptic and postsynaptic structures would require a higher resolution, as obtained by immuno electronmicroscopy. However, by the available means we could not observe a significant colocalization of the dendritic maker and endogenous Bruchpilot, indicating that there are no extensive presynaptic connections in the dendritic calyx. Likewise, previous immuno electronmicroscopy studies of the dendritic calyx showed numerous postsynaptic sites in the dendrites of MB neurons, but no presynaptic sites [56,141]. In summary, these findings may indicate that there are no presynaptic structures in the dendrites of MB neurons, or less than the intensive signal of fluorescently-tagged presynaptic proteins in the dendrites shows. Hence, the detection of fluorescently-tagged presynaptic proteins in the cell bodies or dendrites of MB neurons in young flies most likely accounts for an artifact of excessive Gal4-driven expression. Contrasting the localization of active zone proteins, fluorescently-tagged exocytotic synaptic vesicle protein Rab 3 (Rab3-YFP) exclusively localized to the axons, and also Synaptotagmin 1 (Syt1-GFP) preferentially localized to the axonal compartment. The preferentially axonal localization of exocytotic synaptic vesicle proteins is in line with the axonal localization of Imac-GFP, which is required for axonal transport of Syt1 bearing synaptic vesicles. This further supports a mainly axonal localization of synaptic vesicles and associated proteins and may reinforce the presence of presynaptic structures primarily or exclusively in the axons of *Drosophila* MB neurons. The aberrant detection of Brp-GFP, Cac-eGFP and Synj-eGFP complicated the evaluation of the polarized distribution of presynaptic proteins in the MB of young flies. But the recent study of Nicolai and colleagues using DenMark to characterize the „dendrome“ of four different neuronal populations in the *Drosophila* brain showed that even if dendrites are morphologically distinguishable from axons, they acquire their complete molecular identity relatively late in development [78]. The same may account for axons, which might not have acquired their full presynaptic

specialization in the young stage, leading to the mislocalization of synaptic constituents in MB neurons of young flies. Controversially, proteins involved in anterograde transport of these constituents might more reliably localize to the axon at this stages, as they are increasingly required to achieve the final molecular specialization of the axon. Apparent high expression levels of the transgenic presynaptic proteins Brp-GFP and Cac-eGFP, as well as to a minor degree Syt1-GFP, might have additionally contributed to the mislocalization, as a consequence of excessive protein levels. In accordance with the preferentially axonal localization of Syt1-GFP, Rab3-YFP and the synaptic vesicles transporting Imac-GFP, as well as the antibody staining for endogenous Bruchpilot, this suggests an axonal polarization of presynaptic proteins and thus a classical polarization of presynaptic proteins in the MBs of young flies.

6.2. Maintenance of polarized protein distribution with age

Up to now, studies regarding neuronal polarization have focused on developmental or early stages in life. Surprisingly, the possibility of age-dependent changes in neuronal polarity has never been examined. Thus, having confirmed the axonal polarization of several transgenically expressed proteins in the MBs of young *Drosophila* neurons, we examined the possibility of potential alterations in the polarized distribution of these proteins with age and found differences in the polarized behavior of several proteins with age.

6.2.1. Differential localization of axonally polarized membrane proteins with age

As discussed above, we found transgenic GFP-fusion proteins of the three Drosophila Robo receptor isoforms to preferentially or exclusively localize to the axon in the MBs of young flies. In aged flies, we observed transgenic Robo proteins to behave differentially regarding the maintenance of their localization. Robo2-eGFP, which most reliably localized to the distal axon in young, remained polarily distributed, whereas the distribution of Robo1-eGFP and Robo3-eGFP was notably different. The abundance of both proteins, Robo1-eGFP and Robo3-eGFP, in dendrites, increased while being reduced in the axons, suggesting a shift in the axonal/dendritic ratio of the proteins in the MBs of aged flies. This observation may indicate alterations in selective targeting or retention of these proteins in the axon. Membrane proteins were

found to be retained at their designated location in the plasma membrane by different mechanisms, as it has been shown for the somatodendritic membrane receptor DRL and Robo3 [71]. Localization of DRL was shown to be dependent on dynamin mediated endocytosis, while localization of Robo3 is maintained independent of dynamin [71]. The now observed differential behavior of Robo2-eGFP compared to Robo1-eGFP and Robo3-eGFP might suggest that even proteins that are targeted to the same subcellular compartment, respectively the axon, are retained by different mechanisms, of which one/some might be impaired in aged MB neurons. On the other hand, endogenous Robo proteins are majorly involved in early developmental processes and were shown to be expressed only at basal levels in dorsal root ganglia of the adult rats [83,84], which may as well be true for their expression in *Drosophila* MB neurons. Accurate localization of Robo1-eGFP and Robo3-eGFP could be dependent on their function and the persistent Gal4 induced expression might have caused the altered distribution in aged individuals. But even if these alterations don‘t represent a general rearrangement of the localization of endogenous Robo protein isoforms, it is surprising that Robo1-eGFP and Robo3-eGFP distribution was altered, whereas the localization of Robo 2 was maintained. Potential causes for the alteration of the localization of certain Robo receptor isoforms remain to be elucidated and further research on the mechanisms underlying the polarized distribution of membrane proteins is required to clarify this question.

6.2.2. Maintenance of the polarized localization of motor proteins and adapters with age

In the MBs of young flies, we found the transgenic transport proteins Imac-GFP and Aplip1-GFP to exclusively localize to the axon, with a concentration in the most distal parts of the axons, respectively axon terminals. In aged flies, we could observe no significant alterations in the distribution of Imac-GFP or Aplip1-GFP. Both maintained their highly selective localization to the axon and remained enriched in distal parts of the axons. Imac and Aplip1 are involved in different aspects of axonal transport, as Imac is involved in the delivery of presynaptic components, whereas Aplip1 has broader functions in linking various membranous organelles to Kinesin type 1. The divers functions of these two proteins may allow us to draw broader conclusions on the localization of axon-specific anterograde transport proteins. Consequently, the

unchanged localization of both proteins might indicate an unchanged behavior of proteins involved in axon-specific anterograde transport with age and hence no general alterations of the transport to the axon. Our findings further substantiate the hypothesized role of motor and associated proteins in governing the polarized sorting and trafficking of subcellular components, as they most reliably localized to their designated location within the cell, whereas their associated cargos showed slight mislocalizations, such as Syt1-eGFP. Moreover, we found an age-dependent increase in distal axons for both anterograde transport proteins, potentially indicating an increase in transport to axon terminals. More specifically, the enrichment of Imac-GFP in axon terminals of aged flies points to an increased delivery of presynaptic components as a consequence of synapse maturation and strengthening. On the other hand, a reduced expression of genes involved in vesicle or protein transport, including Dynein and Kinesin, has been shown in the cortex of the aged human brain [142] and a general reduction of axonal transport was reported to proceed pathologic symptoms of several age-associated neurological diseases, including Alzheimer disease [35]. References in *Drosophila* are still missing, but the enrichment of the transgenically expressed transport proteins in the axon may alternatively point to a compensatory mechanism, in which the transgenic proteins become enriched in the axon to compensate for the reduced levels of the endogenous proteins. Then again, it can not be ruled out that the increase in axonal fluorescence was a result of persistent Gal4-driven expression. But this can be considered rather unlikely as both proteins became specifically enriched in distal parts of the axon, whereas no notable increase was observed in other parts of the axon.

6.2.3. Age-dependent changes in the distribution of presynaptic proteins (?)

We found the transgenic presynaptic proteins Brp-GFP, Cac-eGFP and Synj-eGFP to mislocalize to the cell bodies and presumably dendrites of the MBs in young flies. In aged flies, we observed a clear rearrangement in the distribution of these proteins, leading to a preferentially axonal localization and a significant reduction in dendrites and cell bodies. Consistently, the endocytotic synaptic vesicle proteins Syt1-eGFP and Rab3-YFP, which showed a preferentially axonal localization in the young, remained concentrated in the axon, although marginally detectable in the dendrites.

But a more pronounced enrichment of the proteins was observed in the axonal compartment. Taken together with the highly selective axonal localization of Imac-GFP, which is required for axonal transport of Syt1-bearing synaptic vesicles, in aged flies, the age-associated enrichment or preferential localization of synaptic vesicle associated proteins in the axon indicates that the minor localization of the proteins to dendrites can be contributed to persistent Gal4-driven expression and excessive protein levels, leading to a mislocalization of the proteins. Hence, synaptic vesicle proteins can be reasonably assumed to maintain their preferentially axonal localization in aged MB neurons. In summary, all presynaptic proteins, regardless of being associated with the plasma membrane or synaptic vesicles, became preferentially localized to the axonal compartment with age, which is consistent with the previously reported localization of these proteins to axon terminals of motor neurons and photoreceptor cells. Furthermore, the distribution of exogenous presynaptic proteins in aged individuals resembles the distribution of endogenous bruchpilot in young flies. Thus, this distribution is more likely to reflect the distribution of the endogenous proteins, than the rather uniform localization of the transgenic proteins in the young stage.

UAS-insertions of Brp-GFP, Cac-eGFP and Synj-eGFP were highly expressed in the MBs, which is likely to account for the massive accumulation of these proteins in the cell bodies and mislocalization to the dendrites. The reduced levels of the three presynaptic proteins in aged individuals and the preferential localization to the axons might suggest that presynaptic connections are not fully established in the early stages, leading to a retention of the proteins in the cell body and a mislocalization to dendrites. As a consequence of synapse maturation and the reported increase in synapse size with age [143], which presumably requires an increased integration of newly synthesized proteins, the retention may be partially abolished and presynaptic proteins are progressively transported from the cell body to the axon. Then again, expression of proteins involved in synaptic function was shown to decline in the aged brain of *Drosophila melanogaster* and mammals, as shown for Rab 3 in *Drosophila* [144], Rab3A and Syt1 in humans [142]. Moreover, age-associated damages of the Synj promoter, resulting in a reduced expression in the human cortex were shown [142]. Hence, the increase in the axonal localization of transgenic presynaptic proteins and the concurrent reduction in dendrites may alternatively point to an in-

creased recruitment of these proteins to the axon, in order to compensate for the reduction of endogenous proteins in aged individuals, whereas the transport of transgenic presynaptic proteins to the axon might be restricted in the young, as a consequence of protein saturation or competition with endogenous proteins. Again, it can not be ruled out that mislocalization was caused by major limitations of the UAS/Gal4 system. Decreased 201Y-driven expression of the Brp-GFP, Cac-eGFP and Synj-eGFP insertions and thus reduced protein levels might have led to a more reliable localization of these proteins to their designated sites in the axon. No reduction of 201Y-driven expression was evident for any other proteins and most candidate proteins appeared to become enriched in the MBs with age. Thus, this explanation seems rather unlikely to account for the observed alterations in the distribution of presynaptic proteins. However, potential age-dependent alterations in the distribution of presynaptic proteins remain questionable, as we obtained ambiguous results by the available experimental means. Antibody stainings for the endogenous proteins and assessment of endogenous or transgenic protein levels in the MBs of young and aged flies might help to clarify, why transgenic presynaptic proteins tend to mislocalize and whether the observed alterations for transgenic proteins reflect to any degree an alteration in the distribution of endogenous presynaptic proteins.

In summary we found alterations in the polarized distribution of two transgenic membrane proteins, Robo1 and Robo2-eGFP, whereas proteins involved in anterograde axonal transport remained highly polarized, suggesting no general alterations in the polarized trafficking of proteins to the axon. The more surprising are the observed alteration in the distribution of membrane proteins. The mechanisms that govern and regulate the polarized distribution of subcellular components and thus neuronal polarity remain highly elusive, but certainly involve several highly-regulated steps, each of which is likely to be dependent on numerous factors. An impairment in any of these steps would result in various polarity defects, such as targeting or retention of proteins at their destination. Regardless of potential causes and consequences, additional experiments are required to ascertain our findings. Firstly verification of the results in additional age groups might be required to substantiate our findings. Repeating the experiments with young flies that are older than seven days might allow to rule out that the observed localization in the young age group reflects a developmen-

tal stage in which certain aspects of polarity, such as synapse formation, have not been fully established. Moreover, flies older than 45 days, as a control for aged flies to ascertain that the observed distribution reflects indeed the localization in old individuals. As obvious from our experiments, the expression level of a given protein is crucial for its proper localization and overexpression of proteins necessarily leads to a mislocalization of the protein. Thus, a pulsed expression of the proteins in young and aged flies by a combination of the Gal4 system with the Gal80 repressor might be beneficial, to obtain an equal time of expression in each age group and consequently similar protein levels. In any case, antibody stainings to determine the localization of the endogenous proteins in young and old flies are recommendable, to assure that the localization of transgenic proteins reflects the distribution of the endogenous protein. Most studies on protein localization and neuronal polarity in *Drosophila* have exploited the unquestionably useful genetic tools, but as evident from our results, overexpression may easily leads to artifacts and account to a great extent for the confusion in the discussion on the polarity of *Drosophila* neurons.

7. References

1. Foletti D, Prekeris R, Scheller R, Bredt D, Butz S, Okamoto M, Südhof T: **Generation and Maintenance of Neuronal Polarity**. *Neuron* 1999, **23**:641-644.
2. Craig AM, Banker G: **Neuronal polarity**. *Annual review of neuroscience* 1994, **17**:267-310.
3. Mattson MP: **Establishment and Plasticity of Neuronal Polarity**. *Journal of Neuroscience Research* 1999, **57**:577–589
4. Niewiadomska G, Baksalerska-Pazera M: **Age-dependent changes in axonal transport and cellular distribution of Tau 1 in the rat basal forebrain neurons**. *Neuroreport* 2003, **14**:1701-1706.
5. Niewiadomska G, Baksalerska-Pazera M, Riedel G: **Altered cellular distribution of phospho-tau proteins coincides with impaired retrograde axonal transport in neurons of aged rats**. *Ann N Y Acad Sci* 2005, **1048**:287-295.
6. Filipek A, Schneider G, Mietelska A, Figiel I, Niewiadomska G: **Age-dependent changes in neuronal distribution of CacyBP/SIP: comparison to tubulin and the tau protein**. *Journal of Neural Transmission* 2008, **115**:1257-1264.
7. Lai HC, Jan LY: **The distribution and targeting of neuronal voltage-gated ion channels**. *Nat Rev Neurosci* 2006, **7**:548-562.
8. Steward O, Banker GA: **Getting the message from the gene to the synapse: sorting and intracellular transport of RNA in neurons**. *Trends Neurosci* 1992, **15**:180-186.
9. Hevner RF, Wong-Riley MT: **Neuronal expression of nuclear and mitochondrial genes for cytochrome oxidase (CO) subunits analyzed by in situ hybridization: comparison with CO activity and protein**. *J Neurosci* 1991, **11**:1942-1958.
10. Brodin L, Bakeeva L, Shupliakov O: **Presynaptic mitochondria and the temporal pattern of neurotransmitter release**. *Philos Trans R Soc Lond, B, Biol Sci* 1999, **354**:365-372.
11. Mattson MP, Bruce-Keller AJ: **Compartmentalization of signaling in neurons: evolution and deployment**. *Journal of Neuroscience Research* 1999, **58**:2-9.
12. Baas PW, Deitch JS, Black MM, Banker GA: **Polarity orientation of microtubules in hippocampal neurons: uniformity in the axon and nonuniformity in the dendrite**. *Proc Natl Acad Sci USA* 1988, **85**:8335-8339.
13. Burton P: **Dendrites of mitral cell neurons contain microtubules of opposite polarity**. *Brain Research* 1988, **473**:107-115.
14. Matus A, Bernhardt R, Hugh-Jones T: **High molecular weight microtubule-associated proteins are preferentially associated with dendritic microtubules in brain**. *Proc Natl Acad Sci U S A* 1981, **78**:3010-3014.

15. Papasozomenos SC, Binder LI: **Phosphorylation determines two distinct species of Tau in the central nervous system**. *Cell Motil Cytoskeleton* 1987, **8**:210-226.
16. Bradke F, Dotti C: **Membrane traffic in polarized neurons**. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* 1998, **1404**:245-258.
17. Westenbroek RE, Noebels JL, Catterall WA: **Elevated expression of type II Na+ channels in hypomyelinated axons of shiverer mouse brain**. *J Neurosci* 1992, **12**:2259-2267.
18. Horton A, Ehlers M: **Neuronal polarity and trafficking**. *Neuron* 2003, **40**:277-295.
19. Dotti C, Simons K: **Polarized sorting of viral glycoproteins to the axon and dendrites of hippocampal neurons in culture**. *Cell* 1990, **62**:63-72.
20. Dotti CG, Kartenbeck J, Simons K: **Polarized distribution of the viral glycoproteins of vesicular stomatitis, fowl plague and Semliki Forest viruses in hippocampal neurons in culture: a light and electron microscopy study**. *Brain Research* 1993, **610**:141-147.
21. Roman LM, Garoff H: **Alteration of the cytoplasmic domain of the membrane-spanning glycoprotein p62 of Semliki Forest virus does not affect its polar distribution in established lines of Madin-Darby canine kidney cells**. *The Journal of Cell Biology* 1986, **103**:2607-2618.
22. Killisch I, Dotti CG, Laurie DJ, Lüddens H, Seeburg PH: **Expression patterns of GABAA receptor subtypes in developing hippocampal neurons**. *Neuron* 1991, **7**:927-936.
23. Perez-Velazquez JL, Angelides KJ: **Assembly of GABAA receptor subunits determines sorting and localization in polarized cells**. *Nature* 1993, **361**:457-460.
24. Cameron PL, Sudhof TC, Jahn R, De Camilli P: **Colocalization of synaptophysin with transferrin receptors: implications for synaptic vesicle biogenesis**. *J Cell Biol* 1991, **115**:151-164.
25. Fuller SD, Simons K: **Transferrin receptor polarity and recycling accuracy in "tight" and "leaky" strains of Madin-Darby canine kidney cells**. *The Journal of Cell Biology* 1986, **103**:1767-1779.
26. Huttner WB, Dotti CG: **Exocytotic and endocytotic membrane traffic in neurons**. *Current opinion in neurobiology* 1991, **1**:388-392.
27. Jung LJ, Scheller RH: **Peptide processing and targeting in the neuronal secretory pathway**. *Science* 1991, **251**:1330-1335.
28. Tahirovic S, Bradke F: **Neuronal polarity**. *Cold Spring Harbor Perspectives in Biology* 2009, **1**:a001644.
29. Winckler B, Mellman I: **Neuronal polarity: controlling the sorting and diffusion of membrane components**. *Neuron* 1999, **23**:637-640.

30. Dotti CG, Banker GA: **Experimentally induced alteration in the polarity of developing neurons**. *Nature* 1987, **330**:254-256.
31. Geddes JW, Matus AI, Paula ST, Bittar EE: **Chapter 2 The Neuronal Cytoskeleton: Changes Associated with Age, Neurodegenerative Disease, and Neuronal Insult**. In *Advances in Cell Aging and Gerontology*. Edited by: Elsevier; 1997:23-50. Volume 2.
32. Avila J: **The influence of aging in one tauopathy: Alzheimer 's disease**. *Arch Immunol Ther Exp (Warsz)* 2004, **52**:410-413.
33. Mandelkow EM, Mandelkow E: **Tau in Alzheimer's disease**. *Trends in cell biology* 1998, **8**:425-427.
34. Niewiadomska G, Baksalerska-Pazera M, Lenarcik I, Riedel G: **Compartmental protein expression of Tau, GSK-3β and TrkA in cholinergic neurons of aged rats**. *Journal of Neural Transmission* 2006, **113**:1733-1746.
35. Perlson E, Maday S, Fu MM, Moughamian AJ, Holzbaur EL: **Retrograde axonal transport: pathways to cell death?** *Trends Neurosci 2010,* **33**:335-344.
36. Bier E: **Drosophila, the golden bug, emerges as a tool for human genetics**. *Nat Rev Genet* 2005, **6**:9-23.
37. St Johnston D: **The art and design of genetic screens: Drosophila melanogaster**. *Nat Rev Genet* 2002, **3**:176-188.
38. St Johnston D, Ahringer J: **Cell Polarity in Eggs and Epithelia: Parallels and Diversity**. *Cell* 2010, **141**:757-774.
39. Vladar EK, Antic D, Axelrod JD: **Planar cell polarity signaling: the developing cell's compass**. *Cold Spring Harbor Perspectives in Biology* 2009, **1**:a002964.
40. Rolls MM: **Neuronal polarity in drosophila: Sorting out axons and dendrites**. *Developmental neurobiology* 2011, **71**:419-429
41. Rolls MM, Satoh D, Clyne PJ, Henner AL, Uemura T, Doe CQ: **Polarity and intracellular compartmentalization of Drosophila neurons**. *Neural development* 2007, **2**:7.
42. Stone MC, Roegiers F, Rolls MM: **Microtubules have opposite orientation in axons and dendrites of Drosophila neurons**. *Molecular biology of the cell* 2008, **19**:4122-4129.
43. Armstrong JD, Kaiser K, Müller A, Fischbach KF, Merchant N, Strausfeld NJ: **Flybrain, an on-line atlas and database of the Drosophila nervous system**. *Neuron* 1995, **15**:17-20.
44. Shulman JM, Shulman LM, Weiner WJ, Feany MB: **From fruit fly to bedside**. *Current Opinion in Neurology* 2003, **16**:443-449.
45. Margulies C, Tully T, Dubnau J: **Deconstructing memory in Drosophila**. *Curr Biol* 2005, **15**:R700-713.
46. Hallem EA, Carlson JR: **The odor coding system of Drosophila**. *Trends Genet* 2004, **20**:453-459.

47. Tamura T, Chiang A-S, Ito N, Liu H-P, Horiuchi J, Tully T, Saitoe M: **Aging specifically impairs amnesiac-dependent memory in Drosophila**. *Neuron* 2003, **40**:1003-1011.
48. Ye Y, Godzik A: **Comparative analysis of protein domain organization**. *Genome Res* 2004, **14**:343-353.
49. Sánchez-Soriano N, Bottenberg W, Fiala A, Haessler U, Kerassoviti A, Knust E, Löhr R, Prokop A: **Are dendrites in Drosophila homologous to vertebrate dendrites?** *Dev Biol* 2005, **288**:126-138.
50. Katsuki T, Joshi R, Ailani D, Hiromi Y: **Compartmentalization within neurites: Its mechanisms and implications**. *Developmental neurobiology* 2011, 71:458-473.
51. Leiss F, Groh C, Butcher NJ, Meinertzhagen IA, Tavosanis G: **Synaptic organization in the adultDrosophilamushroom body calyx**. *The Journal of Comparative Neurology* 2009, **517**:808-824.
52. Heisenberg M: **What do the mushroom bodies do for the insect brain? An introduction**. *Learning & Memory* 1998, **5**:1-10.
53. Heisenberg M, Borst A, Wagner S, Byers D: **Drosophila mushroom body mutants are deficient in olfactory learning**. *J Neurogenet* 1985, **2**:1-30.
54. Yao Yang M, Armstrong J, Vilinsky I, Strausfeld N, Kaiser K: **Subdivision of the Drosophila mushroom bodies by enhancer-trap expression patterns**. *Neuron* 1995, **15**:45-54.
55. Technau GM: **Fiber number in the mushroom bodies of adult Drosophila melanogaster depends on age, sex and experience**. *J Neurogenet* 1984, **1**:113-126.
56. Yasuyama K, Meinertzhagen I, Schürmann F: **Synaptic organization of the mushroom body calyx in Drosophila melanogaster**. *The Journal of Comparative Neurology* 2002, **445**:211-226.
57. Stocker R, Heimbeck G, Gendre N, de Belle JS: **Neuroblast ablation in Drosophila P [GAL4] lines reveals origins of olfactory interneurons**. *Journal of Neurobiology* 1997, **32**:443-456 .
58. Stocker R, Lienhard M, Borst A, Fischbach, KF: **Neuronal architecture of the antennal lobe in Drosophila melanogaster.** Cell and tissue Res. 1990, **262**: 9-34.
59. Tanaka NK, Tanimoto H, Ito K: **Neuronal assemblies of the Drosophila mushroom body**. *THE JOURNAL OF COMPARATIVE NEUROLOGY* 2008, **508**:711-755.
60. Heisenberg M: **Mutants of brain structure and function: what is the significance of the mushroom bodies for behavior?** *Basic Life Sci* 1980, **16**:373-390.
61. Ito K, Awano W, Suzuki K, Hiromi Y, Yamamoto D: **The Drosophila mushroom body is a quadruple structure of clonal units each of which contains a**

virtually identical set of neurones and glial cells. *Development* 1997, **124**:761-771.

62. Crittenden JR, Skoulakis EM, Han KA, Kalderon D, Davis RL: **Tripartite mushroom body architecture revealed by antigenic markers**. *Learn Mem* 1998, **5**:38-51.
63. Ito K, Suzuki K, Estes P, Ramaswami M, Yamamoto D, Strausfeld NJ: **The organization of extrinsic neurons and their implications in the functional roles of the mushroom bodies in Drosophila melanogaster Meigen**. *Learn Mem* 1998, **5**:52-77.
64. Johard HAD, Enell LE, Gustafsson E, Trifilieff P, Veenstra JA, Nässel DR: **Intrinsic neurons of Drosophila mushroom bodies express short neuropeptide F: relations to extrinsic neurons expressing different neurotransmitters**. *THE JOURNAL OF COMPARATIVE NEUROLOGY* 2008, **507**:1479-1496.
65. Lee T, Lee A, Luo L: **Development of the Drosophila mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast**. *Development* 1999, **126**:4065-4076.
66. Keene AC, Waddell S: **Drosophila olfactory memory: single genes to complex neural circuits**. *Nat Rev Neurosci* 2007, **8**:341-354.
67. Strausfeld NJ, Sinakevitch I, Vilinsky I: **The mushroom bodies of Drosophila melanogaster: an immunocytological and golgi study of Kenyon cell organization in the calyces and lobes**. *Microsc Res Tech* 2003, **62**:151-169.
68. Bullock T, Horridge G: **Structure and Function in the Nervous Systems of Invertebrates**. *San Francisco & London: Freeman* 1965:1-798.
69. Peters A, Palay SL, deF. WH: **The fine structure of the nervous system neurons and their supporting cells.** *New York: Oxford University Press* 1991, **3rd ed.** :407-486.
70. Leiss F, Koper E, Hein I, Fouquet W, Lindner J, Sigrist S, Tavosanis G: **Characterization of dendritic spines in the Drosophila central nervous system**. *Dev Neurobiol* 2009, **69**:221-234.
71. Katsuki T, Ailani D, Hiramoto M, Hiromi Y: **Intra-axonal Patterning: Intrinsic Compartmentalization of the Axonal Membrane in Drosophila Neurons**. *Neuron* 2009, **64**:188-199.
72. Mobbs PG: **Neural networks in the mushroom bodies of the honeybee**. *Journal of Insect Physiology* 1984, **30**:43-58.
73. Rybak J, Menzel R: **Anatomy of the mushroom bodies in the honey bee brain: the neuronal connections of the alpha-lobe**. *THE JOURNAL OF COMPARATIVE NEUROLOGY* 1993, **334**:444-465.
74. Sjöholm M, Sinakevitch I, Strausfeld NJ, Ignell R, Hansson BS: **Functional division of intrinsic neurons in the mushroom bodies of male Spodoptera littoralis revealed by antibodies against aspartate, taurine, FMRF-amide, Mas-allatotropin and DC0**. *Arthropod Struct Dev* 2006, **35**:153-168.

75. Strausfeld NJ: **Organization of the honey bee mushroom body: representation of the calyx within the vertical and gamma lobes**. *THE JOURNAL OF COMPARATIVE NEUROLOGY* 2002, **450**:4-33.
76. Ayaz D: **Axonal growth and regeneration in the Drosophila brain** *Doctoral thesis in Medical Sciences. Katholieke Universiteit Leuven, Leuven, Belgium* 2009.
77. Brand AH, Perrimon N: **Targeted gene expression as a means of altering cell fates and generating dominant phenotypes**. *Development* 1993, **118**:401-415.
78. Nicolaï LJJ, Ramaekers A, Raemaekers T, Drozdzecki A, Mauss AS, Yan J, Landgraf M, Annaert W, Hassan BA: **Genetically encoded dendritic marker sheds light on neuronal connectivity in Drosophila**. *Proceedings of the National Academy of Sciences* 2010, **107**:20553-20558.
79. Morrison JH: **Life and Death of Neurons in the Aging Brain**. *Science* 1997, **278**:412-419.
80. Aso Y, Grübel K, Busch S, Friedrich A, Siwanowicz I, Tanimoto H: **The mushroom body of adult Drosophila characterized by GAL4 drivers**. *J Neurogenet* 2009, **23**:156-172.
81. Hofbauer A: **Eine Bibliothek monoklonaler Antikörper gegen das Gehirn von Drosophila melanogaster.** *Habilitation thesis. University of Würzburg, Würzburg, Germany.* 1991.
82. Seeger M, Tear G, Ferres-Marco D, Goodman CS: **Mutations affecting growth cone guidance in Drosophila: genes necessary for guidance toward or away from the midline**. *Neuron* 1993, **10**:409-426.
83. Marillat V, Cases O, Nguyen-Ba-Charvet KT, Tessier-Lavigne M, Sotelo C, Chédotal A: **Spatiotemporal expression patterns of slit and robo genes in the rat brain**. *THE JOURNAL OF COMPARATIVE NEUROLOGY* 2002, **442**:130-155.
84. Wehrle R, Camand E, Chedotal A, Sotelo C, Dusart I: **Expression of netrin-1, slit-1 and slit-3 but not of slit-2 after cerebellar and spinal cord lesions**. *Eur J Neurosci* 2005, **22**:2134-2144.
85. Dotti CG, Parton RG, Simons K: **Polarized sorting of glypiated proteins in hippocampal neurons**. *Nature* 1991, **349**:158-161.
86. Bunch T, Brower D: **Drosophila cell adhesion molecules.** *Current Topics in Developmental Biology* 1993, **Volume 28**:81-123
87. Rietveld A, Neutz S, Simons K, Eaton S: **Association of sterol- and glycosylphosphatidylinositol-linked proteins with Drosophila raft lipid microdomains**. *J Biol Chem* 1999, **274**:12049-12054.
88. Simons K, Ikonen E: **Functional rafts in cell membranes**. *Nature* 1997, **387**:569-572.

89. Binder L, Frankfurter A, Rebhun L: **The distribution of tau in the mammalian central nervous system.** *The Journal of Cell Biology* 1985, **101**:1371-1378.
90. Goedert M, Wischik CM, Crowther RA, Walker JE, Klug A: **Cloning and sequencing of the cDNA encoding a core protein of the paired helical filament of Alzheimer disease: identification as the microtubule-associated protein tau**. *Proc Natl Acad Sci USA* 1988, **85**:4051-4055.
91. Dehmelt L, Halpain S: **The MAP2/Tau family of microtubule-associated proteins**. *Genome Biol* 2005, **6**:204.
92. Heidary G, Fortini M: **Identification and characterization of the Drosophila tau homolog**. *Mechanisms of development* 2001, **108**:171-178.
93. Drechsel DN, Hyman AA, Cobb MH, Kirschner MW: **Modulation of the dynamic instability of tubulin assembly by the microtubule-associated protein tau**. *Molecular biology of the cell* 1992, **3**:1141-1154.
94. Callahan C, Yoshikawa S, Thomas J: **Tracing axons**. *Current Opinion in Neurobiology* 1998, **8**:582-586.
95. Lo KY, Kuzmin A, Unger SM, Petersen JD, Silverman MA: **KIF1A is the primary anterograde motor protein required for the axonal transport of dense-core vesicles in cultured hippocampal neurons**. *Neuroscience letters* 2011, **491**:168-173.
96. Dajas-Bailador F, Jones E, Whitmarsh A: **The JIP1 scaffold protein regulates axonal development in cortical neurons**. *Current Biology* 2008, **18**:221-226.
97. Horiuchi D, Barkus R, Pilling A, Gassman A, Saxton W: **APLIP1, a kinesin binding JIP-1/JNK scaffold protein, influences the axonal transport of both vesicles and mitochondria in Drosophila**. *Current Biology* 2005, **15**:2137-2141.
98. Goldstein A, Wang X, Schwarz T: **Axonal transport and the delivery of presynaptic components**. *Current Opinion in Neurobiology* 2008, **18**:495-503.
99. Hirokawa N, Takemura R: **Molecular motors and mechanisms of directional transport in neurons**. *Nat Rev Neurosci* 2005, **6**:201-214.
100. Pilling AD, Horiuchi D, Lively CM, Saxton WM: **Kinesin-1 and Dynein are the primary motors for fast transport of mitochondria in Drosophila motor axons**. *Molecular biology of the cell* 2006, **17**:2057-2068.
101. Pack-Chung E, Kurshan PT, Dickman DK, Schwarz TL: **A Drosophila kinesin required for synaptic bouton formation and synaptic vesicle transport**. *Nature neuroscience* 2007, **10**:980-989.
102. Barkus RV, Klyachko O, Horiuchi D, Dickson BJ, Saxton WM: **Identification of an axonal kinesin-3 motor for fast anterograde vesicle transport that facilitates retrograde transport of neuropeptides**. *Molecular biology of the cell* 2008, **19**:274-283.

103. Riemensperger T, Völler T, Stock P, Buchner E, Fiala A: **Punishment prediction by dopaminergic neurons in Drosophila**. *Curr Biol* 2005, **15**:1953-1960.
104. Verhey KJ, Meyer D, Deehan R, Blenis J, Schnapp BJ, Rapoport TA, Margolis B: **Cargo of kinesin identified as JIP scaffolding proteins and associated signaling molecules**. *The Journal of Cell Biology* 2001, **152**:959-970.
105. Taru H, Iijima K-I, Hase M, Kirino Y, Yagi Y, Suzuki T: **Interaction of Alzheimer's beta -amyloid precursor family proteins with scaffold proteins of the JNK signaling cascade**. *J Biol Chem* 2002, **277**:20070-20078.
106. Pellet JB, Haefliger JA, Staple JK, Widmann C, Welker E, Hirling H, Bonny C, Nicod P, Catsicas S, Waeber G, et al.: **Spatial, temporal and subcellular localization of islet-brain 1 (IB1), a homologue of JIP-1, in mouse brain**. *Eur J Neurosci* 2000, **12**:621-632.
107. Craig AM, Blackstone CD, Huganir RL, Banker G: **The distribution of glutamate receptors in cultured rat hippocampal neurons: postsynaptic clustering of AMPA-selective subunits**. *Neuron* 1993, **10**:1055-1068.
108. Fletcher TL, Cameron P, De Camilli P, Banker G: **The distribution of synapsin I and synaptophysin in hippocampal neurons developing in culture**. *J Neurosci* 1991, **11**:1617-1626.
109. Atwood H: **NEUROSCIENCE: Gatekeeper at the Synapse**. *Science* 2006, **312**:1008-1009.
110. Wagh D, Rasse T, Asan E, Hofbauer A, Schwenkert I, Dürrbeck H, Buchner S, Dabauvalle M, Schmidt M, Qin G: **Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in Drosophila**. *Neuron* 2006, **49**:833-844.
111. Kittel RJ, Wichmann C, Rasse TM, Fouquet W, Schmidt M, Schmid A, Wagh DA, Pawlu C, Kellner RR, Willig KI, et al.: **Bruchpilot Promotes Active Zone Assembly, Ca2þ Channel Clustering,and Vesicle Release**. *Science* 2006, **312**:1051.
112. Fouquet W, Owald D, Wichmann C, Mertel S, Depner H, Dyba M, Hallermann S, Kittel R, Eimer S, Sigrist S: **Maturation of active zone assembly by Drosophila Bruchpilot**. *The Journal of Cell Biology* 2009, **186**:129-145.
113. Schürmann F-W: **Bemerkungen zur funktion der corpora pedunculata im gehirn der insekten aus morphologischer sicht**. *Experimental Brain Research* 1974, **19**:406-432.
114. von Schilcher F: **A mutation which changes courtship song in Drosophila melanogaster**. *Behav Genet* 1977, **7**:251-259.
115. Smith LA, Wang X, Peixoto AA, Neumann EK, Hall LM, Hall JC: **A Drosophila calcium channel alpha1 subunit gene maps to a genetic locus associated with behavioral and visual defects**. *J Neurosci* 1996, **16**:7868-7879.

116. Wheeler DB, Randall A, Sather WA, Tsien RW: **Neuronal calcium channels encoded by the alpha 1A subunit and their contribution to excitatory synaptic transmission in the CNS**. *Prog Brain Res* 1995, **105**:65-78.
117. Stanley EF, Mirotznik RR: **Cleavage of syntaxin prevents G-protein regulation of presynaptic calcium channels**. *Nature* 1997, **385**:340-343.
118. Catterall WA: **Structure and function of voltage-gated ion channels**. *Annu Rev Biochem* 1995, **64**:493-531.
119. Kawasaki F, Zou B, Xu X, Ordway R: **Active zone localization of presynaptic calcium channels encoded by the cacophony locus of Drosophila**. *Journal of Neuroscience* 2004, **24**:282-285.
120. Kawasaki F, Felling R, Ordway RW: **A temperature-sensitive paralytic mutant defines a primary synaptic calcium channel in Drosophila**. *J Neurosci* 2000, **20**:4885-4889.
121. Kawasaki F, Collins SC, Ordway RW: **Synaptic calcium-channel function in Drosophila: analysis and transformation rescue of temperature-sensitive paralytic and lethal mutations of cacophony**. *J Neurosci* 2002, **22**:5856-5864.
122. Geppert M, Südhof T: **RAB3 and synaptotagmin: the yin and yang of synaptic membrane fusion**. *Annual review of neuroscience* 1998, **21**:75-95.
123. Kidokoro Y: **Roles of SNARE proteins and synaptotagmin I in synaptic transmission: studies at the Drosophila neuromuscular synapse**. *Neurosignals* 2003, **12**:13-30.
124. Littleton J, Bellen H, Perin M: **Expression of synaptotagmin in Drosophila reveals transport and localization of synaptic vesicles to the synapse**. *Development* 1993, **118**:1077-1088.
125. Wang X, Shaw W, Tsang H, Reid E, O'Kane C: **Drosophila spichthyin inhibits BMP signaling and regulates synaptic growth and axonal microtubules**. *Nature neuroscience* 2007, **10**:177-185.
126. Zhang J, Schulze K, Hiesinger P, Suyama K, Wang S, Fish M, Acar M, Hoskins R, Bellen H, Scott M: **Thirty-one flavors of Drosophila rab proteins**. *Genetics* 2007, **176**:1307-1322.
127. Ng EL, Tang BL: **Rab GTPases and their roles in brain neurons and glia**. *Brain Res Rev* 2008, **58**:236-246.
128. Verstreken P, Koh T, Schulze K, Zhai R, Hiesinger P, Zhou Y, Mehta S, Cao Y, Roos J, Bellen H: **Synaptojanin is recruited by endophilin to promote synaptic vesicle uncoating**. *Neuron* 2003, **40**:733-748.
129. Cremona O, Di Paolo G, Wenk MR, Lüthi A, Kim WT, Takei K, Daniell L, Nemoto Y, Shears SB, Flavell RA, et al.: **Essential role of phosphoinositide metabolism in synaptic vesicle recycling**. *Cell* 1999, **99**:179-188.
130. Gad H, Ringstad N, Löw P, Kjaerulff O, Gustafsson J, Wenk M, Di Paolo G, Nemoto Y, Crun J, Ellisman MH, et al.: **Fission and uncoating of synaptic**

clathrin-coated vesicles are perturbed by disruption of interactions with the SH3 domain of endophilin. *Neuron* 2000, **27**:301-312.

131. Harris TW, Hartwieg E, Horvitz HR, Jorgensen EM: **Mutations in synaptojanin disrupt synaptic vesicle recycling**. *The Journal of Cell Biology* 2000, **150**:589-600.
132. Dickman D, Lu Z, Meinertzhagen I, Schwarz T: **Altered synaptic development and active zone spacing in endocytosis mutants**. *Current Biology* 2006, **16**:591-598.
133. Wang J, Ma X, Yang JS, Zheng X, Zugates CT, Lee CH, Lee T: **Transmembrane/juxtamembrane domain-dependent Dscam distribution and function during mushroom body neuronal morphogenesis**. *Neuron* 2004, **43**:663-672.
134. Lowenstein P, Morrison E, Douglas P, Preston C, Clissold P, Banting G, Stow N, Castro M: **Neocortical neuronal polarity: targeting of a foreign protein linked to a glycosylphosphatidylinositol (GPI) anchor in postmitotic neurons and polarized distribution of a marker of the trans-Golgi network (TGN 38).** *Biochemical Society transactions* 1993, **(2)**:117S.
135. Greco V, Hannus M, Eaton S: **Argosomes: a potential vehicle for the spread of morphogens through epithelia**. *Cell* 2001, **106**:633-645.
136. Feuillette S, Miguel L, Frébourg T, Campion D, Lecourtois M: **Drosophilamodels of human tauopathies indicate that Tau protein toxicityin vivois mediated by soluble cytosolic phosphorylated forms of the protein**. *Journal of Neurochemistry* 2010, **113**:895-903.
137. Estes PS, Ho GL, Narayanan R, Ramaswami M: **Synaptic localization and restricted diffusion of a Drosophila neuronal synaptobrevin--green fluorescent protein chimera in vivo**. *J Neurogenet* 2000, **13**:233-255.
138. Santos JG, Vomel M, Struck R, Homberg U, Nassel DR, Wegener C: **Neuroarchitecture of peptidergic systems in the larval ventral ganglion of Drosophila melanogaster**. *PLoS One* 2007, **2**:e695.
139. Zhong L, Hwang RY, Tracey WD: **Pickpocket is a DEG/ENaC protein required for mechanical nociception in Drosophila larvae**. *Curr Biol 2010,* **20**:429-434.
140. Roy B, Singh AP, Shetty C, Chaudhary V, North A, Landgraf M, Vijayraghavan K, Rodrigues V: **Metamorphosis of an identified serotonergic neuron in the Drosophila olfactory system**. *Neural Dev* 2007, **2**:20.
141. Leiss F, Groh C, Butcher NJ, Meinertzhagen IA, Tavosanis G: **Synaptic organization in the adultDrosophila mushroom body calyx**. *The Journal of Comparative Neurology* 2009, **517**:808-824.
142. Lu T, Pan Y, Kao S, Li C, Kohane I, Chan J, Yankner B: **Gene regulation and DNA damage in the ageing human brain**. *Nature* 2004, **429**:883-891.

143. Beramendi A, Peron S, Casanova G, Reggiani C, Cantera R: **Neuromuscular Junction in Abdominal Muscles of Drosophila melanogaster During Adulthood and Aging**. *The Journal of Comparative Neurology* 2007, **501**:498–508.
144. Kim S, Rhee J, Song Y, Park D, Hwang M, Lee S, Kim J, Gim B, Yoon J, Kim Y: **Age-dependent changes of gene expression in the Drosophila head**. *Neurobiol Aging* 2005, **26**:1083-1092.

8.Appendix

8.1. Abbreviations

Aplip1	App-Like-Protein Interacting-Protein 1
App	Amyloid-Precursor-Protein
ATP	Adenosintriphosphate
AZ	Active zone
Brp	Bruchpilot
Ca^{2+}	Calcium ions
Ca^{2+}-channels	Calcium ion channel
Cac	Cacophony
CAST	CAZ-Associated Structural Protein
CNS	Central Nervous System
Cyo	Curly O (Balancer chromosome)
DenMark	Dendritic Marker
dephospho-Tau	Dephosphorylated Tau protein
Dscam	*Drosophila* Down Syndrome Cell Adhesion Molecule
eGFP	Enhanced Green Fluorescent Protein
ERC	ELKS Rab3- Interacting Protein CAST
GABA	γ-Aminobutyric Acid
GFP	Green Fluorescent Protein
GFPgpi	Green Fluorescent Protein fused to GPI
GPI	Glycosylphosphatidylinositol
GTP	Guanosine-5'-triphosphate
Imac	Immaculate Connections
JIP 1	Janus Kinase Scaffolding Protein 1
JNK	Janus Kinase
K^{+}	Kalium Ion

Khc	Kinesin Heavy Chain
Kif	Kinesin Superfamily Protein
Kif1bβ	Kinesin Superfamily Protein bβ
LNv	Lateral Neuron Ventral (Ventral Lateral Neurons)
MAP	Microtubule Associated Protein
MB	Mushroom Body
mCD8	Mouse Cluster of Differentiation 8
MT	Microtubule
mTau	Mouse Tau protein
Na^+	Sodium Ion
NgCam	Neuron Glial Cell Adhesion Molecule
Pax-DG	Protein Blocking Solution
PBS	Phosphate Buffer Saline
PBT	Phosphate Buffer Saline containing Triton
PN	Projection Neurons
Rab3	Ras-like Protein in Rat Brain 3
Robo	Roundabout Receptor
SFV-E	Semiliki Forest Virus Glycoprotein E
Synj	Synaptojanin
Syt1	Synaptotagmin 1
UAS	Upstream Activating Sequence
VSV-G	Vesicular Stomatitis Virus Protein G
YFP	Yellow Fluorescent Protein

8.2. Acknowledgments

First I would like to thank Carlos Dotti, who gave me the opportunity to carry out this project in his laboratory and provided an excellent scientific environment for my thesis. I will always value his encouraging energy and love for science. I owe special thanks to my supervisor at the University of Vienna, Michael Kiebler, for supporting me throughout my studies and helping me with all organizational issues regarding my thesis.

Furthermore, I would like to thank particularly my supervisor Joachim Schulz who trained me and taught me how to handle flies, brains and lab life. This project would not have been possible, without his great support and his patience in answering my questions regarding the theoretical and practical outline of this project.

Special thanks to Giulia Pollarolo for her interest in my thesis, as well as her personal and scientific support. The discussions with her and her critical remarks, have been invaluable for this work.

I would also like to thank all my other colleagues from the laboratory of Neuronal Differentiation, especially Leen van Huffel for her encouragement and practical support, Antonio Laranjeirea for helping me with the dissection, Isabelle Lahousse for organizational and personal support, Tina Whale and Mauricio Martin for advice at lunch, as well as Sebastian Munck and Wouter Vanormelingen for help with the microscopy.

Last but not least I would like to thank my family and friends for their outstanding support and never ending belief in me.

8.3.Curriculum Vitae

PERSONAL DETAILS

Name	Penelope Dimas
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Citizenship	Austria

EDUCATION

2008 - 2011University of Vienna, Vienna
- *Second level studies in molecular Biology (equivalent to masters)*

2005 - 2008 University of Vienna, Vienna
First state examination (equivalent to a Bachelor) with honors
- *First level studies in molecular Biology*

1996 - 2004 Bundesrealgymnasium Klusemann, Graz
Matura with honors
- *Grammar school*

1996 - 2004 Volksschule Krones, Graz
- *Elementary school*

WORK EXPERIENCE

February - October 2010 Catholic University of Leuven, Leuven
Practical work for the diploma thesis in the group of Carlos Dotti, Department of Human genetics, Unit for Neuronal Differentiation

August 2009 Max F. Perutz Laboratories, Vienna
Internship in the group of Cecile Brocard

November – January 2009 Center of Brain Research, Vienna
Lab rotation training

July – August 2008 Center of Brain Research, Vienna
Internship in the group of Michael Kiebler

SCHOLARSHIPS

February – August 2010
Förderungsstipendium of the University Vienna
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SKILLS

- Spoken languages:
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  - Spanish, French (basic)

- Full computer literacy in word processing, spreadsheet, and graphic presentation packages